THE FLUOROMETRIC ANALYSIS OF HUMAN CHROMOSOMES

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By

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This book is dedicated

to Lida and Riana.

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I N T R O D U C T I O N

The importance of chromosomes as the site of hereditary traits was only recognized at the end of the nineteenth century. It was then learned that in a given species the number of chromosomes in each cell are constant, and the shape of each individual chromosome is characteristic of that species. The cytological interpretation of the Mendelian laws of heredity followed and gave rise to the founding of modern cytogenetics. (Sutton, 1903; De Vries 1903; Boveri, 1904). Further studies of cell division and genetic phenomena led to the discovery of sex determination (McClung, 1902, Stevens, 1905) and the demonstration of gene linkage (Morgan, 1910, 1911).

Arnold (1879) was probably the first person to attempt the determination of the number of chromosomes in human cells. Flemming in 1882 described and pictured mitoses from normal human tissue. Some twenty large and small chromosomes are recognizable in a division figure of a corneal cell. Winiwater reported in 1912 that 47 elements were present in testes of males and 48 in ovaries of females. In 1917, Wiemann reported the presence of X and Y chromosomes in man. Painter (1921) concluded that 48 chromosomes were the characteristic diploid number in both sexes. This was the general belief until 1956 when Tjio and Levan described the first clinically usable method for the observation, characterization and accurate counting of chromosomes. They consistently found 46 chromosomes in human somatic cells.

In 1949 Barr and Bertram published their observation of a distinctive chromatin mass limited to the female, in the nuclei of the motor neurons of the hypoglossal nucleus of the mature cat

brain. By 1953 Moore, Graham and Barr had found that human female cells could be distinguished from male cells in skin biopsies. The more useful oral smear method was introduced by Moore and Barr in 1955.

The application of these two independent techniques to clinical problems, has proved to be an usefull diagnostic tool. These advances in the field of human cytogenetics have provided new insights into the aetiology and pathogenesis of many human disorders associated with mental and/or somatic maldevelopment. Cytogenetic techniques have played a major role in uncovering causative factors in developmental abnormalities of the human reproductive system.

To date much of human cytogenetics has been concerned with identifying and examining persons with abnormal karyotypes and the knowledge of incidences is limited. Chromosomal aberrations, detectable at mitosis, are not uncommon and are remarkably frequent in certain syndromes.

Until recently human chromosomes were stained for identification with staines, which coloured the chromosomes preferentially. The only means using these techniques, of distinguishing between one chromosome and another are by the length of the chromosomes and position of the centromere. Identification of chromosomes was limited in that the 22 pairs of autosomes and the X and Y sex chromosomes could only be classified into 10 distinct groups. The accuracy of detection of structural heterozygotes from the analysis of mitotic cells therefore have important limitations. Paracentric inversions cannot be detected and the only pericentric inversions detectable are those following breaks non-equidistant from the centromere, causing a visually recognizable change in the arm

ratio. Reciprocal translocations will go unrecognized where nearly equal amounts of material are exchanged. Simple deletions and duplications can be suspected only when relatively large amounts of material are involved. This inefficiency of recognition has been a major barrier, impeding the study of chromosome damaging agents, particularly in radiation cytogenetics.

The value of the Barr-body, is limited to the identification of individuals with an abnormal number of X chromosomes. In females with a sufficently large structural aberration of one X chromosome, this would also be reflected in the size of the sex-chromatin body. The disadvantage of the Barr-body technique, as the primary means of assessment in examining the male population, is that it fails to detect those with an abnormal sex chromosome complement involving only a single X chromosome,

It has been shown recently that some fluorescent dyes, such as quinacrine mustard, stain certain chromosome regions in a specific fashion. Caspersson and associates in studying human chromosomes, first observed a strong fluorescent region in the Y chromosome, and then described a pattern of bands of fluorescence in the other chromosomes. (Zech, 1969, Caspersson et al 1970a). They further demonstrated that the entire human karyotype can be classified by combining the methods based on chromosome size and centromere position with the fluorescent banding patterns.

The application of this fluorescent banding technique to obtain accurate identification of the normal human chromosomes under routine laboratory conditions, has been motivation for the present study. A system for recording the fluorescence patterns, suitable also for computerized analysis of large numbers of chromosomes has been developed and evaluated.

The presence of the bright fluorescence region of the Y chro-

mosome also in nuclei of non-dividing cells was observed by Pearson et al (1970) and George (1970), using quinacrine derivatives. This test seems to complement the Barr-body test in that abnormal Y-chromosomes in males could be detected in the easily obtained interphase cells.

To test the validity of the use of quinacrine derivatives in nuclear sexing, individuals with abnormal Y-chromosome complements were examined and the results used to determine the incidence of Y-chromosome abnormalities in lymphocytes of the newborn population.

The variation in length of the human Y-chromosome in the normal population has been recognized since the earliest routine analysis of chromosomes. Studying the Y-chromosome after staining with quinacrine mustard Zech (1969), revealed two distinct regions of fluorescence: a light fluorescent region as well as an intense fluorescent region. Measurements were made in the present study of Y-chromosomes of normal males to examine the length variation in this chromosome with respect to these two regions.

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C H A P T E R I

THE DEVELOPEMENTS IN THE IDENTIFICATION OF HUMAN CHROMOSOMES

In 1921, Painter saw 46 chromosomes in metaphase of human gonadal tissue but two years later he reported that the human nucleus contains a diploid number of 48 chromosomes, 23 paired autosomes and 1 pair of sex chromosomes. It was generally held that there are a diploid number of 48 chromosomes in human nuclei until 1956 when Tjio and Levan reported 46 chromosomes in somatic cells. This observation was confirmed by the findings of Ford and Hamerton (1956) in testis tissue, Ford et al (1958) in bone marrow cells, and in preparations of skin tissue and facia by Tjio and Puck (1958)

The study of human chromosomes and its application to the medical field, was impossible until recently because of the technical problems involved. The large number of chromosomes, their small size, overlapping as well as their tendency to clump, made the study of the chromosomes a formidable task. Hsu (1952) and Hughes (1952) rediscovered the effect of a hypotonic solution on growing cells, which had been described earlier by Slifer (1934). Under the osmotic effects of the hypotonic solution the cells swell and this promotes separation of metaphase chromosomes.

The chromosomes can be seen as visual entities only during cell dividion. The chromosomes are best and most easily studied at the metaphase stage of mitosis. It was found that incubating growing cells in a medium containing colchicine, for short periods

Figure 1: (a) Normal human male chromosomes stained with Giemsa.
(b) The same cell stained with quinacrine mustard.

before swelling and fixation, provided a high yield of cells in mitotic metaphase. (Ford and Hamerton, 1956, Hsu, 1952 and Tjio and Levan, 1956).

The culturing of leutocytes obtained from peripheral blood was shown by Hungerford et al (1959) and Moorhead et al (1960) to be a rapid and reliable method for doing a chromosome analysis. After Nowell's (1960) discovery that phytohemagglutinun promotes the division of leukocytes in vitro, it became possible to obtain suitable chromosome preparations after a 3-day culture of peripheral blood. An air-drying technique, described by Rothfels and Siminovitch (1958), has been found by Moorhead et al (1960) to spread the chromosomes in a single layer. For selective staining of the chromosomes a number of stains have been used. Methods have been described using Schiff's reagent, aceto arcein, Giemsa solutions and others; all giving a clear, even stain of the chromosomes in metaphase. Figure 1a shows normal chromosomes in metaphase stained with Giemsa.

At present the methods used to study and identify the human chromosomes, utilize cultures from different tissues of the body. Tissues most frequently employed are skin, bone marrow and peripheral blood.

The study of chromosomes in prophase and metaphase of meiosis yields useful information: and techniques have been developed to study chromosome behaviour in testis and ovarian tissues. The application of this technique however, is limited because these tissues are not always easy to obtain.

With these advances in technical skill, a new dissipline, Clinical Cytogenetics has rapidly developed.

Chromosome morphology.

The metaphase chromosomes of human tissues are subdivided into three morhological forms according to the position of the centromere or primary constriction. The position of the centromere is, median in a metacentric chromosome, sub-median in a submetacentric chromosome or in a subterminal position in a acrocentric chromosome. The submedian and subterminal centromere divides the chromosome in two parts, known as the short arms and long arms. The centromeric index expressed as the ratio of the length of the shorter arm to the total length of the chromosome is often used as a diagnostic character of the chromosome. The size of the chromosome, the position of the centromere and the presence of secondary constrictions are important characteristics in the identification of normal human chromosomes.

Different parts of a chromosome may give different staining reactions, with conventional stains such as orcein or Giemsa and others. The heterochromatic regions in the human karyotype are those found to show variation in the staining properties. Euchromatin is that part of a chromosome which exhibits no variation in its staining properties.

Karyotype analysis and Denver report.

The term 'karyotype' refers to the systematic array of the entire chromosome complement of a cell.

The normal human karyotype of mitotic chromosomes was standardized in 1960 by a group of Cytogeneticists during a symposium

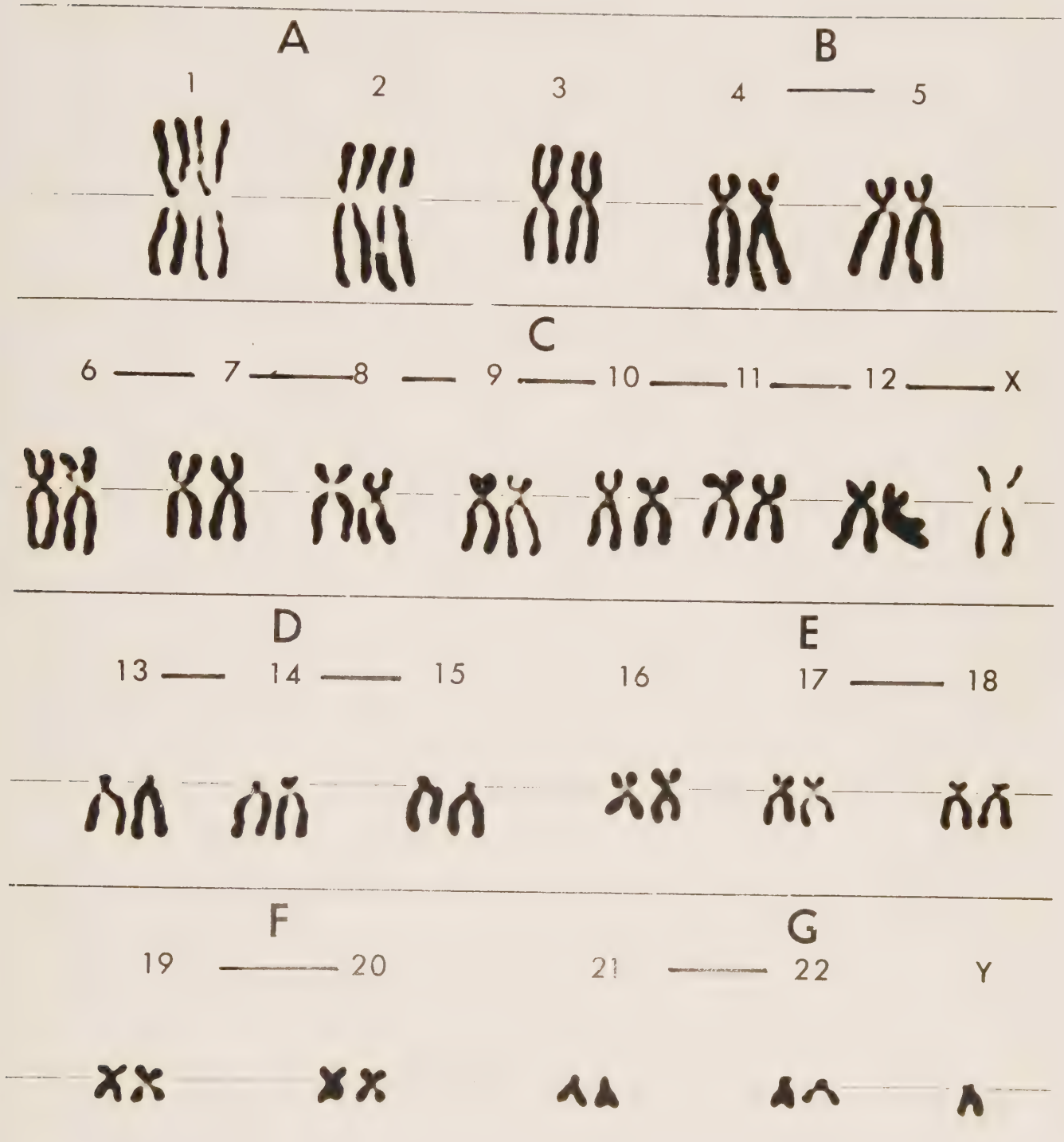

Figure 2 - Karyotype of normal human male chromosomes.

in Denver, U.S.A. The autosomes were numbered from 1 to 22 in descending order of length. The sex chromosomes are referred to as X and Y and are placed near to, but separated from, the groups of autosomes they resemble (figure 2). The autosomes were classified into seven groups and these were outlined by The Human Chromosome Study Group (1960) as follows:

Group 1 - 3	Large chromosomes with approximately median centromeres. The three chromosomes are readily distinguished from each other by size and centromere position.
Group 4 - 5	Large chromosomes with submedian centromeres. The two chromosomes are difficult to distinguish, but chromosome 4 is slightly longer.
Group 6 - 12	Median-sized chromosomes with submedian centromeres. The X-chromosome resembles the longer chromosomes in this group, espesially chromosome 6, from which it is difficult to distinguish. This large group is the one which presents the greatest difficulty in identification of individual chromosomes.
Group 13 - 15	Medium-sized chromosomes with nearly terminal centromeres ("acrocentric" chromosomes). Chromosome 13 has prominent satellites on the short arms. Chromosome 14 has small satellites on the short arms. No satellites have been detected on chromosome 15.

Group 16 - 18	Rather short chromosomes with approximately median (in chromosome 16) or submedian centromeres.
Group 19 - 20	Short chromosomes with approximately median centromeres.
Group 21 - 22	Very short, acrocentric chromosomes. Chromosome 21 has satellites on its short arms. The Y-chromosome is similar to these chromosomes.

The distinction between the chromosomes within certain groups is difficult (figure 2) and conclusive identification of conventionally stained chromosomes is only possible for chromosomes 1, 2, 3 and 16. Patau (1960), independently from the Denver report, designated letters of the alphabet to the seven groups of chromosomes from A to G. He included the X-chromosome with group C and the Y-chromosome with group G.

Secondary constrictions and the London conference.

A conference on the Normal Human Karyotype was called in London in 1963 to consider developments since the Denver Conference and to assess the degree to which these may have aided characterization of individual chromosomes. There was general agreement that the seven groups in the Denver numbering system had stood the test of time, and that little or no uncertainty existed in the group assignment of any chromosome. The most important newly recognized features discussed at the Conference were secondary constrictions and differential patterns of incorporation of isotopically labelled thymidine in specific chromosomes as demonstrated by autoradiography.

The constrictions in chromosomes include the primary or centromeric constrictions and a number of secondary constrictions. The latter do not appear to be equivalent in structure or function. Secondary constrictions are heterochromatic regions on the chromosome (Darlington and La Cour 1941), and these regions are believed to be actively associated with the formation of the cell nucleolus (Heitz, 1931). The nucleolar organizing regions (SAT region), deliminating the satellite from the remainder of the chromosome, are probable functionally distinct from the remainder of the constrictions which are generally interstitial in a chromosome arm. The latter are constant in position but not always visible. Special techniques have been developed to enhance the showing up of these regions (Saksela and Moorhead, 1962, Yunis 1965, and others) but without consistently good results. When they are visible, they are useful markers for chromosome identification.

An agreement was reached at the Conference on the following specific points concerning chromosomes in the 7 groups of mitotic human chromosomes, which were in future to be designated by a capital letter as well as by the first and last number in the group:

Group 1 - 3(A)	In many cells a secondary constriction can be observed in the proximal region of the long arm of no.1. (De La Chapelle, 1961, Miller et al 1962, Saksela and Moorhead, 1962).
Group 4 - 5(B)	No additional information was available to assist in distinguishing between these two chromosome pairs.
Group 6 - 12 and X(C)	Of the autosomes in this group, four are comparatively metacentric. It was proposed that they should be numbered 6, 7, 8 and 11. The X-chromosome belongs to this subgroup. Three chromosomes are submetacentric. They should be numbered 9, 10 and 12.

	A secondary constriction is found in the proximal part of the long arm of at least one of the pairs in the whole group. (Ferguson-Smith *et al*, Muldal and Ockey, 1961). In normal females, one chromosome characteristically incorporates isotopically labelled thymidine over most of its length later than the others in the group. This is believed to be an X chromosome (Morishima *et al* 1962, German, 1962 and Gilbert *et al* 1962).
Group 13 - 15(D)	Satellites have now been detected in all three chromosome pairs. (Ferguson-Smith and Handmaker, 1961 and Ohno *et al*, 1961).
Group 16 - 18(E)	A secondary constriction has frequently been seen in the proximal part of the long arm of no.16. (Saksela and Moorhead, 1962, Sasaki and Makino 1963).
Group 19 - 20(F)	No additional information was available to distinguish between these two chromosome pairs.
Group 21 - 22 and Y(G).	Satellites have now been detected on both nos. 21 and 22. The most common Y-chromosome is larger than either 21 and 22, its centric constriction is often indistinct and a secondary constriction is frequently seen in the long arm. Chromatids of the Y appear to diverge less less than those of the other chromosomes. Size variability is observed in the Y-chromosome and heritable polymorphism has also been observed in chromosomes with satellites and probably in no. 16.

Autoradiography and the Chicago Conference.

The discovery that DNA replication of different chromosomes and of regions of individual chromosomes is asynchronous, provides a further means of distinction between them (Howard and Pelc, 1953). By labelling the DNA precursor, thymidine with a suitable

radioactive isotope, tritium, the uptake of thymidine can be studied in chromosomes by autoradiography of the cells at metaphase. German (1962), Morishima et al (1962), Moorhead and Defendi, (1963), Schmid, (1963) and others showed that these observations have provided a valuable tool for characterizing and identifying many individual chromosomes.

In the report of the Chicago Conference (1966) on the standardization in Human Cytogenetics new means were considered for describing normal chromosomes and deviations from the normal complement. The following notes on autoradiography were published:

> In chromosome no. 1 the distal segment of one arm, here defined as the short arm, terminates DNA synthesis earlier than the remainder of the chromosome. The other arm, here defined as the long arm, often has a secondary constriction near the centromere. There is a short proximal portion of the long arm which often synthesizes DNA relatively late.
>
> In group 13-15 three patterns of termination of synthesis are sometimes recognizable, and these have tentatively been used to specify the three pairs of chromosomes. Two chromosomes, here called pair no. 13, continue synthesis relatively late in extensive segments of their long arms; two chromosomes, here called pair no. 14, complete synthesis relatively late in regions near the centromere; and two chromosomes, here called pair no. 15 generally complete synthesis very early.
>
> In chromosome nos. 17 and 18 the pair here termed no. 17 usually appear to have a more median centromere and are often longer. They terminate synthesis earlier than the two chromosomes of pair no. 18.

Chromosome nos. 19 and 20 are amongst the earliest in the complement to terminate DNA synthesis. Autoradiographic features to distinguish between them have not been described.

All X-chromosomes in excess of one generally replicate later than all the other chromosomes. As a rule, structurally abnormal X-chromosomes (rings, isochromosomes or those with deletions) are also late-replicating The Y-chromosome is relatively late-replicating, especially in comparison with the other chromosomes of the G-group.

Fluorescent banding patterns on human chromosomes.

Caspersson and co-workers (1968) employed the alkylating agents, quinacrine mustard and the antimalarial quinacrine, to test the hypothesis that the presence of a fluorescent alkylating agent was detectable on the chromosomes by ultra-microfluorescence studies.

The biological action of alkylating agents is postulated to be an attack at the N-7 atom of guanine in the DNA. The alkylating agents quinacrine mustard and quinacrine, both fluorescent in the visible range, were found in Vicia faba to interact with DNA by intercalation in the double helix and by ionic binding to the phosphate groups. (Caspersson, 1969a). Quinacrine mustard, in addition, binds to DNA through the alkylating group which probably reacts with guanine of DNA. Caspersson et al (1970b) showed that human chromosomes treated with these agents show a clear pattern of light and dark crosstriations and fluorescent patterns when measured with a special fluorometer with a high resolving power.

Caspersson et al (1969a, 1969b) studied the staining behaviour of a number of fluorochromes and found that fluorescent alkylating agents give superior results than fluorochromes without the alkylating groups.

Other investigatiors, George (1970), Vosa (1970), O'Riordan et al (1971) and others, soon confirmed the studies of Caspersson et al on human chromosomes in metaphase and interphase cells by using quinacrine as the fluorochrome. Caspersson et al (1970j) pointed out several advantages to using quinacrine mustard in preference to quinacrine for fluorescence studies. They found that the bands produced by quinacrine were less clear, less stable and possibly less reproducable, and they also fade rapidly on continued irradiation with ultra-violet light.

The fluorescence pattern, therefore, in a chromosome treated with quinacrine mustard is determined by the amount of DNA in the different regions of the chromosomes as well as by the qualitative differences in the binding capacity of the DNA.

Techniques were developed to record the fluorescent patterns of chromosomes, either directly in the fluorescence microscope (Caspersson et al, 1969a) or on photographs. (Caspersson 1970b, Pawlowitzski, 1971). By using these techniques all 24 different chromosome types of the normal human karyotype could be identified and were found to be highly reproducable.

Caspersson (1971a) used a series of 11 pattern curves from each of the 8 chromosome types in the C-group to show that these pattern types differed significantly. They indicated that the pattern curves can be characterized by a low number of Fourier parameters, making automatic recognition and classification feasible.

Towards the end of April 1971, an international group of Cyto-

logists met in Stockholm to consider the available evidence on the possibility of identifying human chromosomes by fluorescence. Identification proved to be reliable and this report was adopted by the Fourth Standardisation Conference for Human Chromosomes held in Paris in September 1971. (Pearson 1972). The report was not yet available at time of writing of this thesis.

Giemsa banding techniques.

Over the past few months alternative banding techniques have been described for the identification of human chromosomes.

In their studies of the hybridization of radioactive nucleic acid with DNA, Pardue and Gall(1970) also observed that the regions adjacent to the centromeres of the mouse chromosomes stained deeply with Giemsa. They found that mouse satellite DNA is located in the centromeric heterochromatin of the chromosomes. Arrighi and Hsu (1971) modified the denaturing and renaturing techniques of Pardue and Gall and consistently demonstrated the heterochromatic regions in the chromosomes of man. These regions are believed to be composed of constitutive heterochromatin and are almost all localized in the neighbourhood of the centromeres. The amount of heterochromatin, however, is not constant in all chromosomes. The preparations studied by Arrighi and Hsu (1971) showed large heterochromatic regions on the long arm of chromosome no. 1, the D-group chromosomes, no. 16 and on the short arm no. 18. The Y chromosomes heterochromatin was found to be localized in the distal portion of the long arm rather than in the centromeric regions. Some of these regions were shown to coincide with the secondary constriction sites on the chromosomes.

Sumner et al (1971), while repeating the technique of Pardue and Gall (1970) found that their control cells without the denaturing agent also showed marked bands on the chromosomes. The bands obtained by their ASG (ascetic/saline/giemsa) technique and by the quinacrine fluorescence technique appeared to be similar except for the Y chromosomes. The distal part of the long arms of the Y chromosome fluoresces brilliantly with quinacrine and when the ASG technique is used the Y chromosome usually has two bands on its long arms. The other chromosome pairs were found to differ in banding patterns so that each chromosome could be identified.

Seabright (1972) used proteolytic enzyme digestion procedures and produced a series of deeply stained bands on human chromosomes with little or no intervening stained material between the bands, after staining with Giemsa. The chromosome pairs could be identified by their specific banding sequence.

It is not yet clear what the chemical basis is of either the fluorochrome or the denaturation - renaturation Giemsa and ASG techniques.

These new techniques will be of immediate practical value in chromosome identification. During the ten years since the first identification of individual human chromosomes, the only methods of distinguishing morphologically similar chromosomes have been either tedious autoradiography or the study of features such as secondary constrictions, enhanced by special treatments. The scope of cytogenetics has been extended by the two new techniques - the staining with fluorochromes and the use of Giemsa stain after various pretreatments. Preliminary reports show that the two techniques can give rather similar patterns.

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C H A P T E R II

THE VISUAL IDENTIFICATION OF HUMAN CHROMOSOMES BY FLUORESCENT BANDING PATTERNS.

Introduction.

Since the discovery of banding patterns on human chromosomes, a vast amount of detail on the human karyotype has been published. Some reports have claimed that the entire human karyotype can now be identified by studying these banding patterns on individual chromosomes. Most of the methods make use of the visual analysis of each chromosome. The qualitative and quantitative demands of karyotyping are now so great that these visually oriented methods have become inadequate. Visual analysis of banding patterns is limiting because of difficulty in memorizing the patterns.

Aids to visual identification have thus been devised to improve chromosome identification. Length measurements and calculated centromere indexes of the chromosomes are used, for karyotyping Giemsa stained chromosomes. In order to obtain the banding patterns of fluorescent chromosomes Caspersson et al (1970) and Pawlowitzki (1971) used a reflection photometer which could be moved along the length of each chromosome to give a wave pattern.

In order to test the validity of the fluorescence banding technique, chromosomes of 50 normal individuals were stained with quinacrine mustard and examined. The banding patterns on the

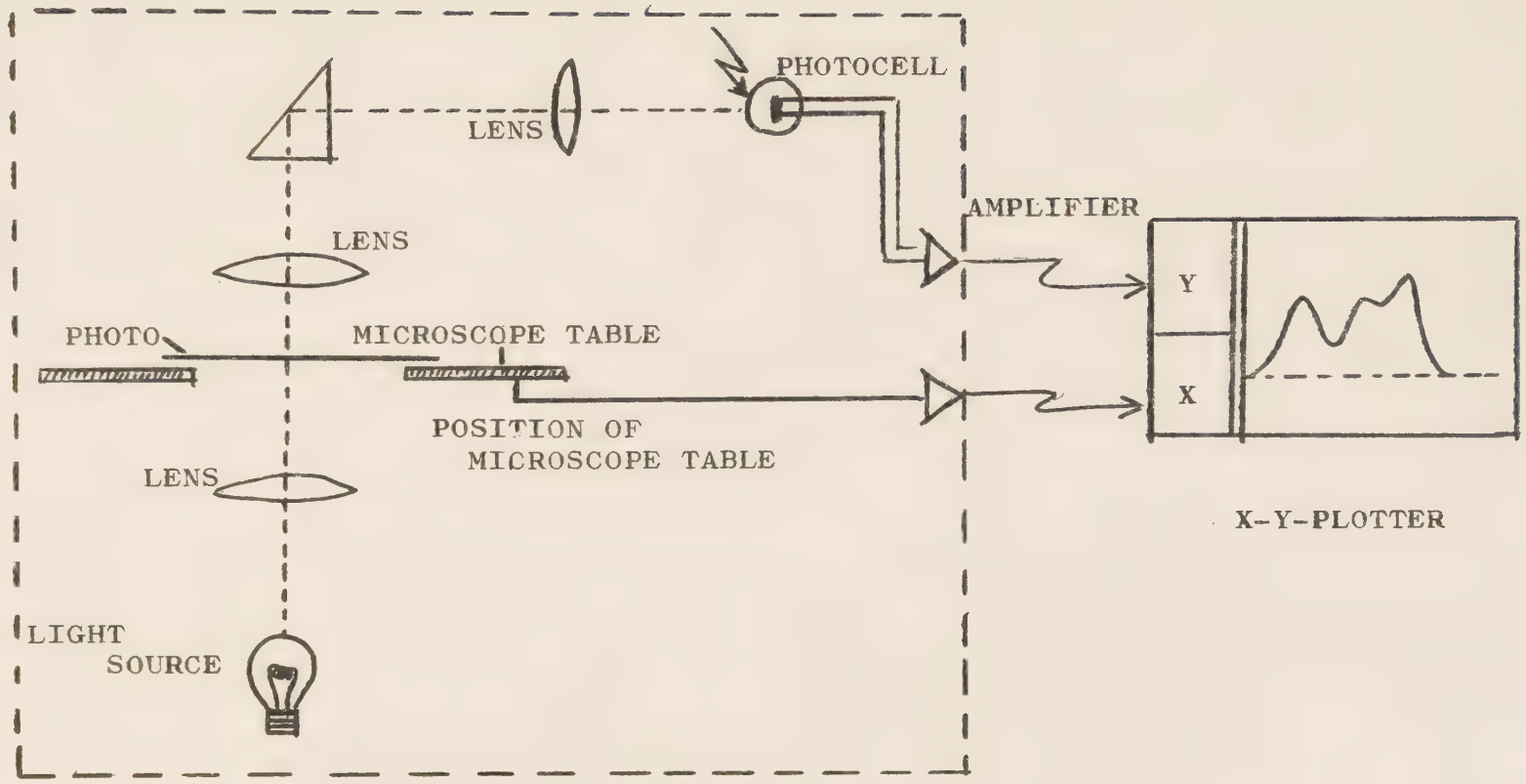

Figure 3 - Schematic representation of the apparatus for the recording of a wave pattern of the fluorescence bands on chromosomes in a photographic print.

chromosomes were recorded photo-electrically and the wave patterns were used as an aid to visual identification of the chromosomes in the metaphase.

Experimental procedures.

(i) Staining: Metaphase chromosomes of blood cultures from 30 normal males and 20 normal females were prepared according to the method of Moorhead et al (1960). The slides were transferred from absolute ethanol through alcohol steps into a solution of Mac Ilvaine's buffer at pH 6.8. The slides were then stained for 10 - 20 minutes in a 50µgm/ml water solution of quinacrine mustard, washed in buffer and dried before covering with fresh buffer and a coverslip sealed with clear nail varnish.

(ii) Microscopy: The slides were examined as soon as possible after staining under a Zeiss fluorescence microscope, using BG 12 exiter and 53/44 barrier filters. Well-spread metaphase figures of chromosomes with lengths that has been used routinely for karyotyping were photographed as soon as they were recognized. Prolonged exposure to ultra-violet rays causes rapid fading and loss of banding detail on the photographs.

(iii) Photography: A total of 300 fluorescent metaphase figures were photographed using Agfa Geavert 35 mm film (45 C 62) and developed in Rodinol. Kodak Tri X and Panatomic X also gave good results. Exposure times varied from 10-40 seconds depending on film speed and overall intensity of fluorescence. The metaphase chromosomes were printed on high-contrast paper. Selected photo-

graphs for photo-electric measurements were printed on transparent paper (Agfa Geavert TP3).

(iv) Photo-electric recording.

Apparatus: A microscope with 2.5X objective, a photo-cell, camera lucida attachment and a X-Y recorder were assembled so that pattern recordings of the banding on the chromosomes could be make. The photo-cell is built into the camera lucida attachment to measure the light intensity and is connected to the Y axis of the recorder (figure 3). The stage movement is connected to the X-axis of the X-Y recorder to convey movement to the X-axis of the recorder.

Method of pattern recordings: A fairly straight and non-overlapping, chromosome is selected from a photograph and aligned so that with stage movement, the light can pass through one of the chromatids along the length axis of the chromosome. A recording of the chromatid is drawn on the X-Y recorder with the stage movement. The scale on the X-Y recorder can be adjusted to fit onto the graph paper used and a conveniently sized graph was drawn of the intensity of each chromosome.

Not all photographs were suitable for fluorometric recording. Of the 300 photographs about one-third were considered suitable and selected for pattern recordings. Chromosomes of different cells exhibited a wide variation in intensity. Some chromosomes stained well, showing clear differences in the bands, in contrast to other chromosomes, sometimes on the same slide, that showed poor differentiation. Generally, similarity of staining was found in chromosomes within the same cell.

Visual identification of chromosomes.

Length measurements, centromere position and the fluorometric patterns described by Caspersson et al (1971a) were used to classify the chromosomes into 24 pattern groups corresponding to numbers 1 to 22 in addition to the X and Y chromosomes of the normal human karyotype.

The presence of satellites on the acrocentric chromosomes and their polymorphic nature make fluorescence measurement studies for comparisons of this segment very difficult. It was decided therefore to disregard the short-arm intensity measurements and to use the only reliable parameter the long-arm patterns.

An average of 50 chromosomes were classified in each group. For the identification of chromosome numbers 8, 10, X, 14 and 15 an elimination technique had to be applied in some cells in order to classify them. Some chromosomes in these last-mentioned groups could not be visually classified with certainty.

The fluorescence patterns of the normal chromosomes have been described by Caspersson et al (1971), Evans et al (1971) and Manolov et al (1971). Caspersson et al and Evans et al described the banding pattern on chromosomes of average length that has been used routinely for karyotyping. Manolov et al on the other hand, did not present photographs of the chromosomes used for their banding studies, but states that the banding details described by them can be seen only in long chromosomes. The use of long chromosomes in karyotyping, however, is very limited. Chromosomes in early metaphase overlap and do not lie straight enough to be measured photo-electrically. Difficulties therefore arise in karyotyping

all the chromosomes in one cell. The identification of only parts of the chromosomes in a cell may lead to erroneous results in the application of this technique.

Nomenclature: The intensity of fluorescent bands on the chromosomes have been described by Caspersson et al (1971) and Evans et al as being dark, dull, even, uniform, strong, high, bright and very bright. The position of these bands were described as being proximal, distal and in the middle of either the long or the short arm.

In their article on banding patterns Manolov et al (1971) mentioned that the nomenclature they proposed followed, in general, the recommendations put forward by the Conference for Standardizing in Human Cytogenetics.

In naming the bands they use Arabic numerals starting from the centromere which is labelled 0 and going from the centromere in both directions. The numbers of the short arm are preceded by p, those of the long arm by q. In the fluorescing karyotype the centromeric regions are dark. If the dark region extends to the short or long arm it is named p0 or q0 until succeeded by the first light band. The first light bands are called p1 and q1 and extend to the next dark bands p2 and q2. As a result dark bands will always have even numbers and light bands odd numbers.

Manolov et al (1971) mentioned that the designation of shades of fluorescence is essentially the same as in the Report, namely:

Negative shades	Dark	---, --	Positive shades Light +
	Dim	-	Bright ++
			Intense +++

The dark shade is thus symbolized by both 2 and 3 minus signs, the latter according to Manolov et al (1971), needed in describing some centromeric regions. Plus and minus in parenthesis denote intermediate shades.

To define as accurately as possible the location of the fluorescent bands Manolov et al (1971) made use of the measurements compiled by Penrose (1964), recalculated as percentages of the female haploid set. The centromere represents the zero point, and percentage distances are measured in both directions from the centromere. Points on a chromosome are localized by means of single percentage values and distances are localized by the percentage values of their two end points. It is possible to express the pattern of fluorescence of each chromosome as a code fraction with the code for the short arm as numerator and that for the long arm as denominator. The formula for chromosome no. 1 will according to Manolov et al (1971), for an example, be:

$$\frac{0\ 1\ 2\ 3\ 4\ 5\ 6\ (6)\ 7\ 8\ (8)\ 9}{0\ 1\ (1)\ 2\ 3\ 4\ (4)\ 5\ 6\ (6)\ 7\ 8\ 9\ (9)}$$

In the present study the bands recognized on the chromosomes are, with a few exceptions, fewer than those seen by Manolov et al (1971). The reason for the difference is that the chromosomes used in this study were of relatively the same size that is currently in use in the laboratory and elsewhere for karyotype analysis. Longer chromosomes overlap and are for this reason difficult to measure photo-electrically. Observations of the bands

were made by taking photo-electric measurements and recording the wave pattern. Manolov et al (1971) visually scanned long chromosomes for the presence of bands and the shades of intensity were subjectively decided upon.

The nomenclature proposed by Manolov et al (1971) was found to be to refined and comprehensive when describing the banding patterns of more contracted chromosomes used for karyotyping. The subjective designation of shades of positive fluorescence into 6 categories and more was impossible even with the aid of photo-electric measurements. It is however, important to know the banding pattern in as much detail as possible to reveal structural abnormalities that is invisible by other methods.

In the present study the intensity and location of bands are described in the following terms:

Intensity:	Positive banding:	Light
		Bright
		Intense
	Negative banding:	Dark
		Dull
Location:	Short arm:	Proximal to the centromere
		Median " " "
		Distal " " "
	Long arm:	Proximal to the centromere
		Median " " "
		Distal " " "

The 24 fluorescent patterns of the Human Karyotype.

A series of each of the 24 normal human chromosomes stained

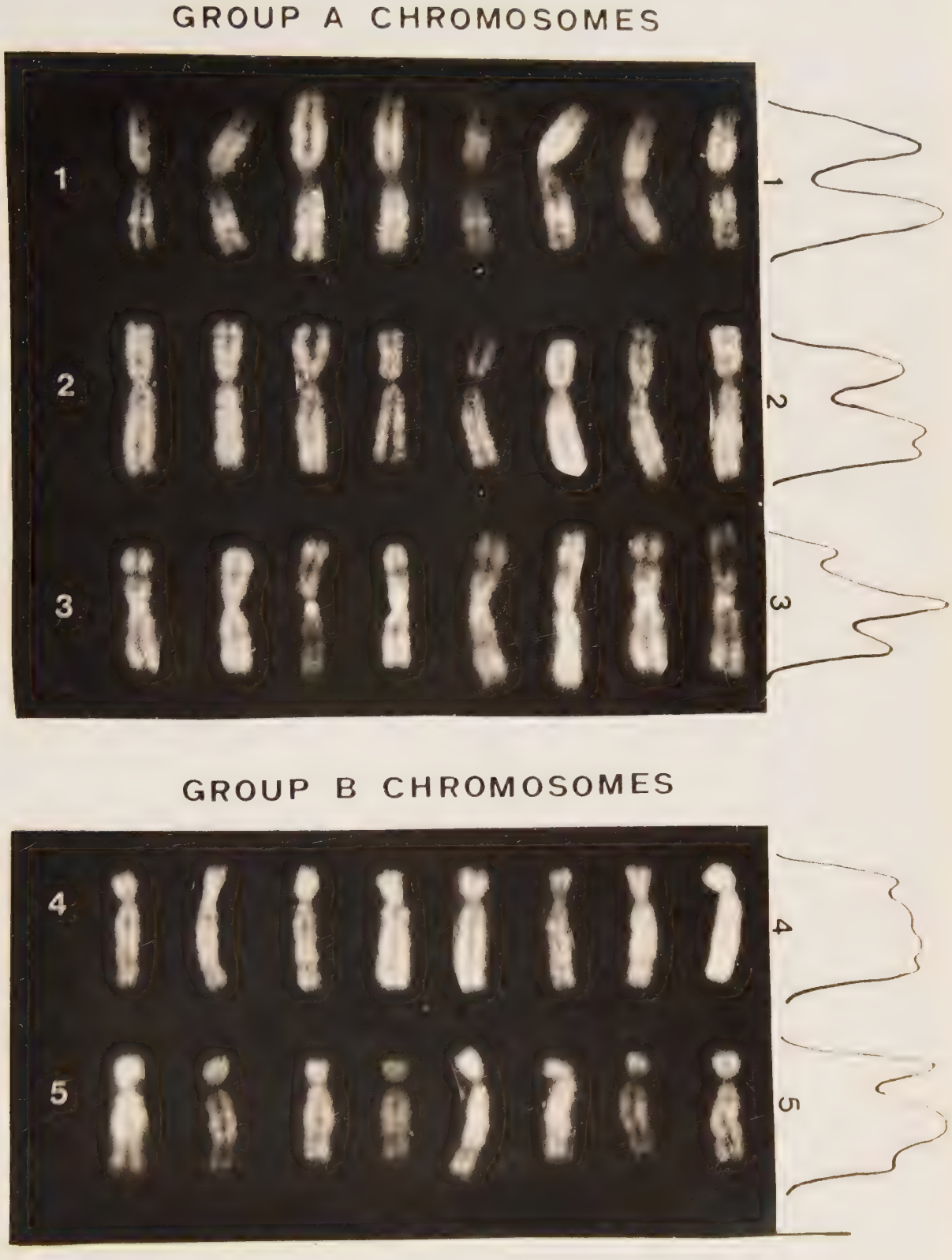

Figure 4 - A series of A and B group chromosomes stained with quinacrine mustard. The average wave pattern recordings are given alongside the chromosomes.

Figure 5 - A series of C-group chromosomes stained with quinacrine mustard. The average wave pattern recordings are given alongside the chromosomes.

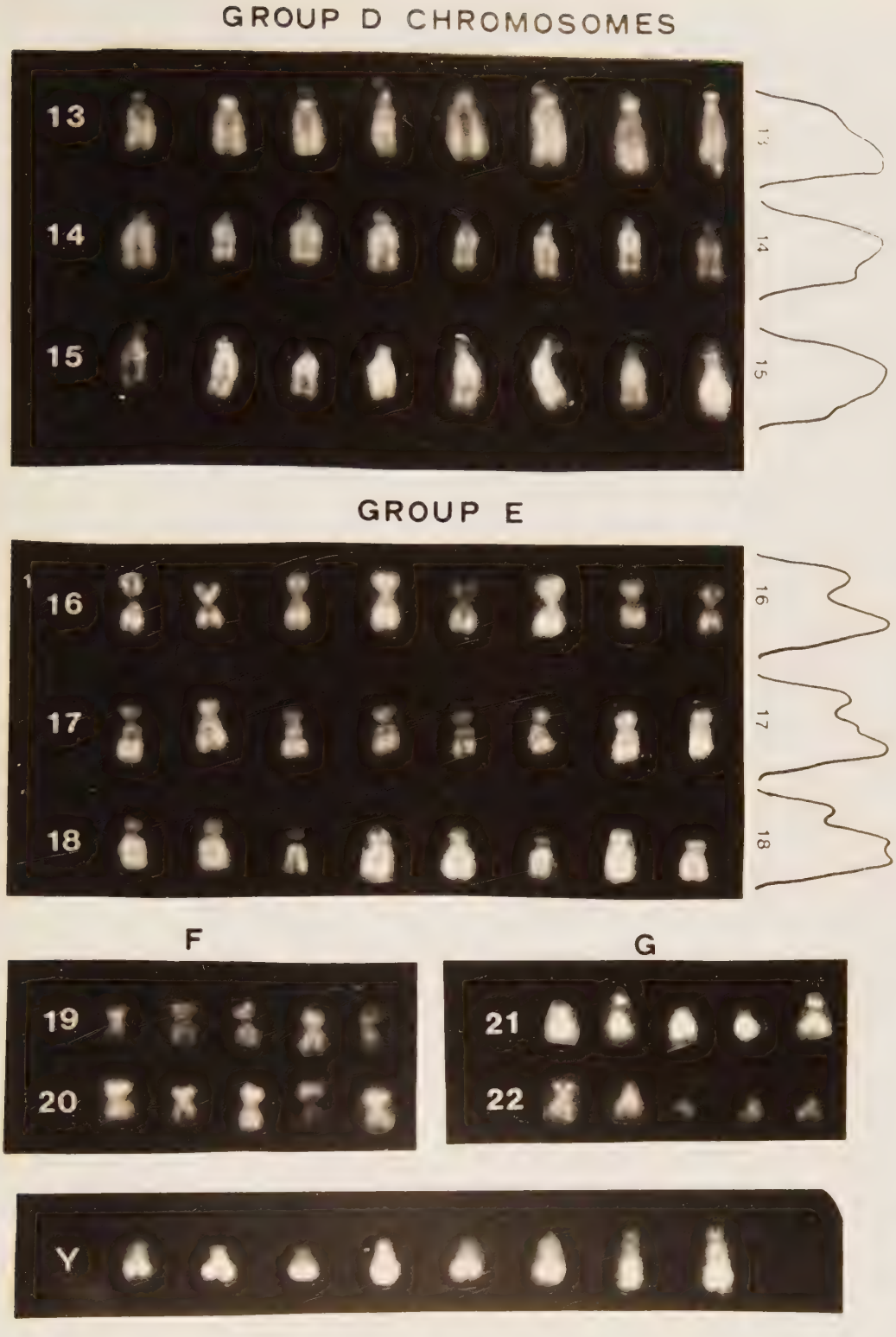

Figure 6 - The D, E, F and G group chromosomes stained with quinacrine mustard with the average wave pattern recordings in groups D and E given alongside.

with quinacrine mustard and the average wave patterns recorded for each group are given in figures 4 - 6. Wave pattern recordings, of a series of examples of each of the 24 normal fluorescent chromosomes are given in figures 7 - 26. The pattern recordings standardized for lengths are made from the short arm telomere to the long arm telomere.

GROUP 1 - 3(A).

The 3 chromosome pairs 1, 2 and 3 of the A-group chromosomes are readily distinguished from the rest of the chromosomes in the karyotype on size and centromere position. Stained with quinacrine mustard the fluorescent patterns are striking and characteristic for each chromosome.

Chromosome number 1.

The number 1 chromosome is the longest chromosome in the karyotype, and is easily recognized using the conventional staining methods. The centromere is in a median position and the short and long arms are of nearly similar lengths. A secondary constriction is frequently observed in the proximal region of the long arm. This region is known to be polymorphic in its coiling state. Families have been described who exhibit an elongated region ~~on~~ in this part of the chromosome and it was referred to, in mapping studies, as the uncoiler locus (un-locus) by Donahue et al (1968).

In the fluorescence studies, the short arm was found to have a bright fluorescent region proximal to the centromere followed by a dull fluorescent region on the distal part. In most of the cells

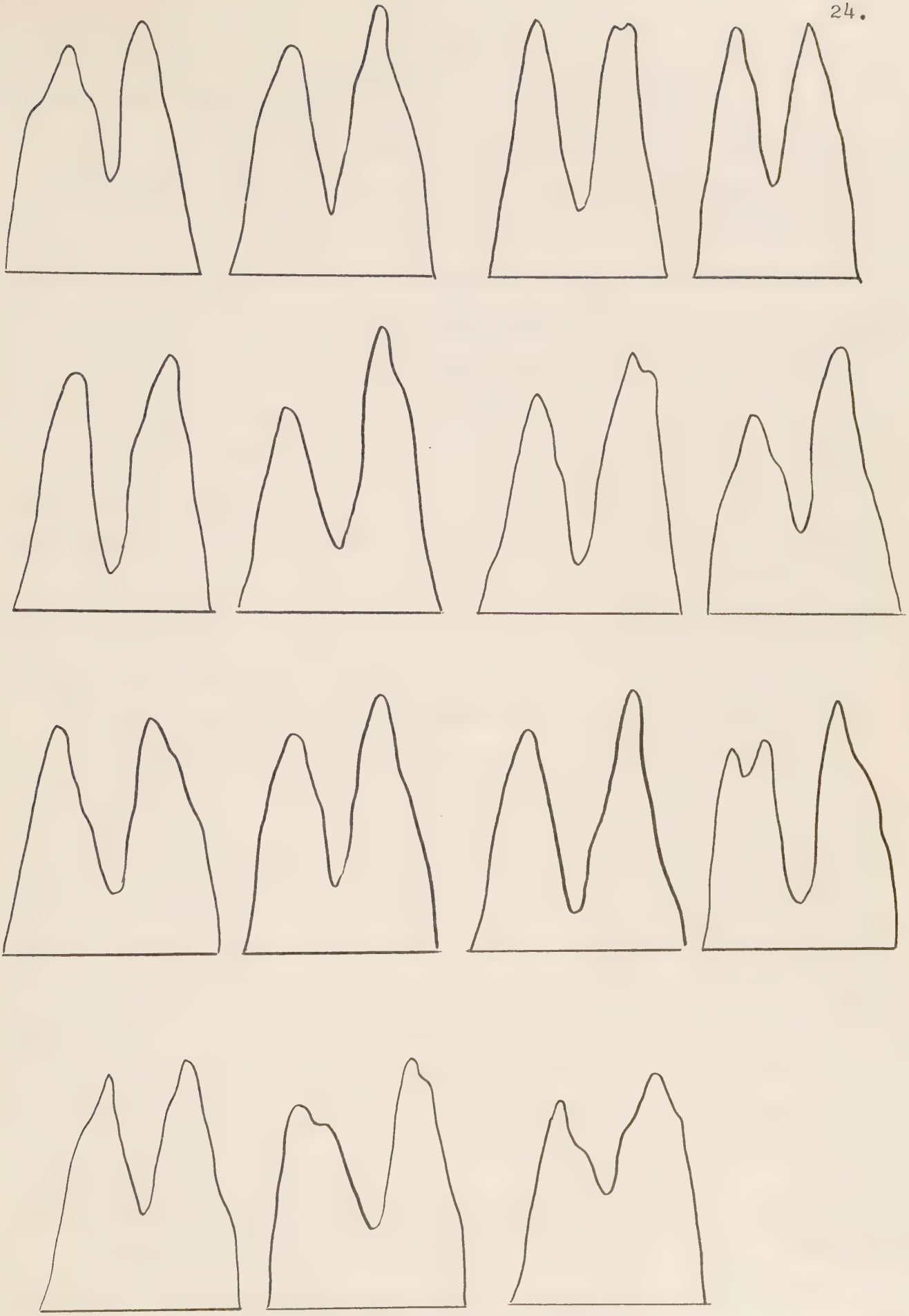

Figure 7 - Wave pattern recordings of 15 fluorescent no. 1 chromosomes.

studied, the bright region showed two bright bands close to each other with about the same intensity measurements. (Figure4). The two bands are those described by Manolov et al (1971) in long chromosomes as p3 and p5. These authors have described another 3 smaller, positive, light fluorescent bands on the short arm in position p1, p7 and p9. Caspersson et al (1971) and Evans et al (1971) have described only two bands proximal to the centromere, with the distal region dull fluorescing.

In autoradiography studies of the bright regions vs. the dull region on the short arm of no. 1, Ganner and Evans (1971) have shown that there is a tenfold difference in grain density between these regions, with a very heavily labelled fluorescent region. These studies show a correlation between positive fluorescence and a late DNA replication pattern.

In good Giemsa banding preparations, the bands formed coincide with those seen by the fluorescence methods. (Evans et al 1971).

The long arm has a negative, dull fluorescing area proximal to the centromere, corresponding to the secondary constriction area. Bright fluorescent bands appear in the median region with a light fluorescing band in the distal region. In longer chromosomes up to 3 positive bands could be recognized. The average pattern shows a high peak in the median region of the long arm (figure 7). This median peak corresponds to q5, and stains brighter than the two more distal bands, q7 and q9. Manolov et al (1971) also described two lighter bands in position q1 and q3.

In karyotyping a more contracted chromosome, the bright median-proximal region adjacent to a dull distal region are characteristic of the short arm. On the long arm, a dull proximal region

and a bright median band with lighter distal bands are consistently found.

With the Giemsa-staining methods, the secondary constriction area on the proximal region of the long arm stains heavily and is polymorphic. The short arm stain with 2 positive bands and the long arm with 4 to 5 bands. (Evans et al 1971).

Chromosome number 2.

The chromosome no. 2 is the second largest chromosome in the karyotype and can easily be identified on the basis of size and a submedian centromere. This large submetacentric chromosome has an overall bright fluorescence after staining with quinacrine mustard, and only the centromere region appears dull fluorescing. Secondary constrictions have not consistently been found in this chromosome.

The short arm of this chromosome fluoresces evenly with the proximal region, having the highest intensity measurements. (band p1). Manolov et al (1971) described 4 positive bands on the short arm with very narrow negative bands in between. The even fluorescence in the short arm of more contracted chromosomes is therefore caused by the coalescence of the 4 brighter bands.

In Giemsa stained preparations, Evans et al (1971) also recognized 4 darker staining bands on the short arm.

The long arm of no. 2 chromosome fluoresces rather evenly (figure 4) with light fluorescence near the centromere and has the highest values around the median to distal region. This brighter region corresponds to band q7 of Manolov et al (1971) described 5 positive bands on the long arm. Caspersson et al (1971) pointed

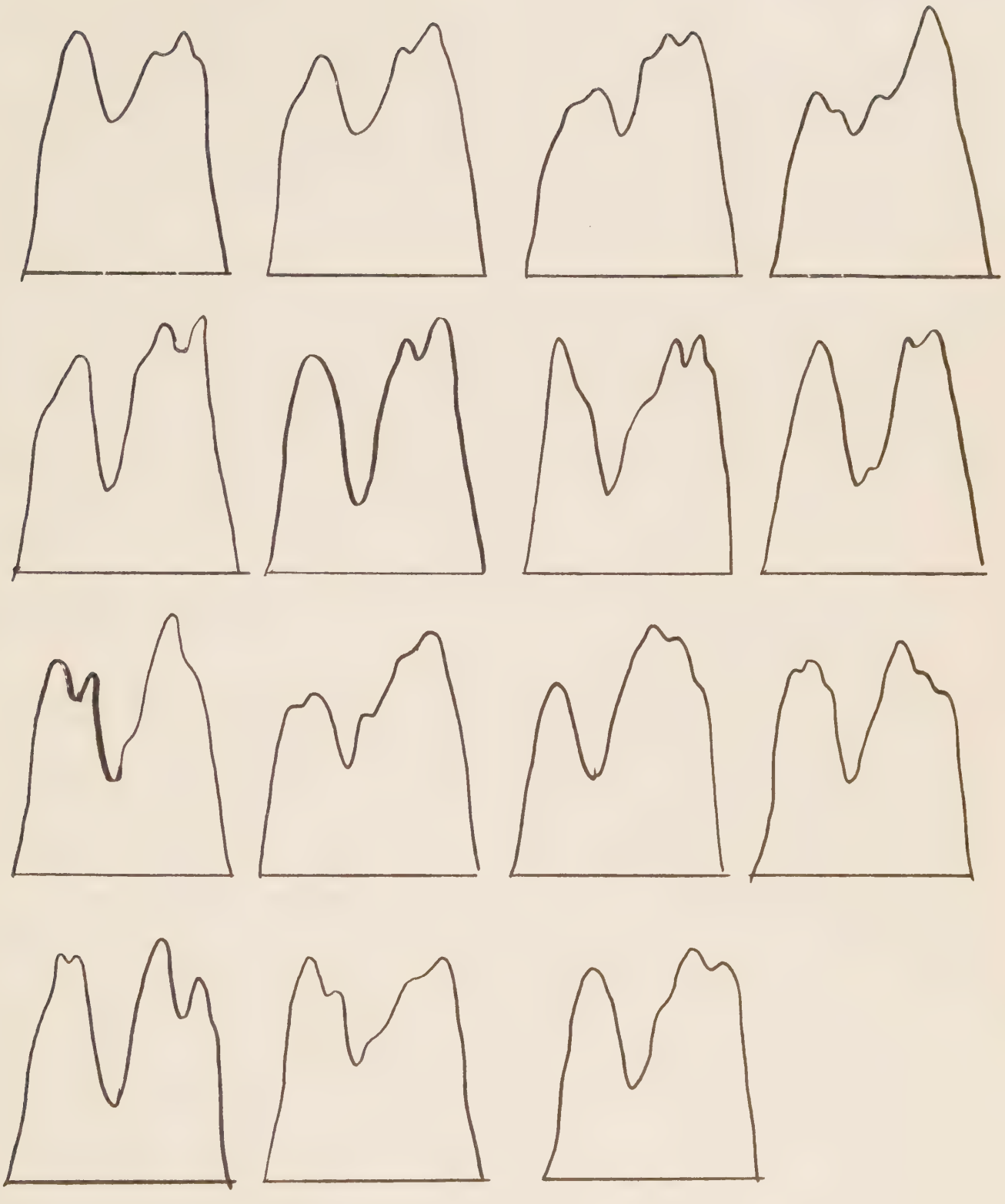

Figure 8 - Wave pattern recordings of 15 fluorescent no. 2 chromosomes.

out 2 definite positive bands on the long arm in photographic prints that showed excellent contrasts.

In Giemsa preparations, up to seven bands were distinguished on the long arm. (Evans et al 1971). In the DNA synthesis patterns of the autosomes compiled by Gianelli (1970), it is interesting to note that the whole of the no. 2 chromosome completes DNA synthesis fairly late.

Identification of chromosome number 2 on the metaphase is easy on the basis of length and centromere studies. With quinacrine mustard staining, this chromosome fluoresces rather featurelessly with an even pattern over the chromosome, except for the centromere region, which appears dull fluorescing.

Chromosome number 3.

The chromosome no. 3 can be identified as being the second largest metacentric chromosome and the third longest chromosome. The only other normal chromosome of nearly similar length and morphology is number 1, which is about 15 to 20% longer than no. 3.

Stained with quinacrine mustard, one or both no. 3 chromosomes are very easy to identify in the metaphase of some individuals. The striking feature in many preparations is the intense fluorescent band near the centromere, which can be identified at first glance (figure 4). This feature does not appear in all no.3 chromosomes and is a polymorfic condition. In 42% of individuals both no. 3 chromosomes had this intense band and in 38% of individuals it appeared on only one no. 3 chromosome. In 20% of individuals no intense band was seen on either chromosome. The size of these bands varies between individuals. Manolov et al (1971) described

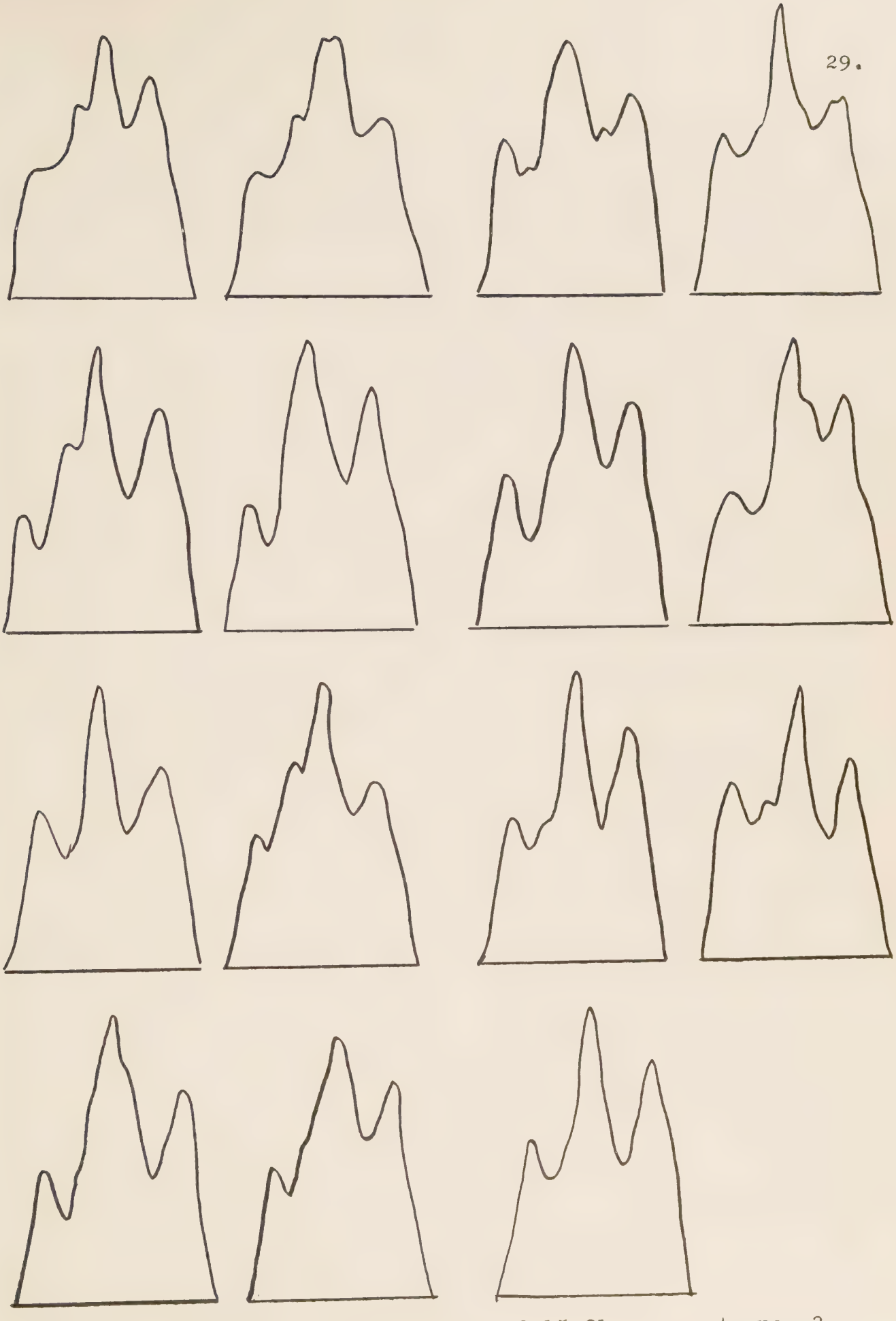

Figure 9 - Wave pattern recordings of 15 fluorescent, no. 3 chromosomes.

these intense stained regions as consisting of two bodies in long chromosomes. In preparations where the chromatids are widely separated these bands are seen as a characteristic of each chromatid and not a feature of the centromere region.

The short arm of the number 3 chromosome stains with a bright proximal band, a dull median region and a light fluorescing distal band. Manolov et al (1971) described 4 positive bands, two bands p1 and p3 proximal and a bright (++) broad distal band p5, followed by a small light band p7 in the telomeric region. From intensity measurements on the wave patterns as seen in figure 9, band p5 differs in the present study as having a light (+) fluorescence in all the no.3 chromosomes studied. Caspersson et al (1971) described the short arm fluorescence as a dark median region surrounded by broad light bands.

The Giemsa staining methods show good agreement with the fluorescent bands, with two dark staining regions proximal and distal on the short arm (Evans et al, 1971).

The long arm of the no. 3 chromosome stains with a pattern nearly identical to that of the short arms. A bright band is seen proximal, with a dull median region and a bright distal region. The bright distal region, bands q5 and q7, have intensity that is higher on photo-electric measurements than that of the p5 region of the short arm. Manolov et al (1971) as well as Caspersson et al (1971) describe these two regions as being of nearly equal intensity. In this study of the no. 3 chromosome, band p5 has been disignated as light in fluorescence and band q5 and q7 as bright fluorescing, as seen on the wave pattern measured photo-electrically. (Figure 9).

In the DNA replication studies of the autosomes, Gianelli (1970) showed a fairly symmetrical pattern of DNA synthesis in the no. 3 chromosome at the end of the S, or DNA synthesis period of the cell cycle. The median parts of both arms are the first to complete DNA synthesis, followed by the distal regions. The centromere and its proximal regions are the last to synthesize DNA. There is evidently a parallel between late DNA synthesis and positive staining with quinacrine in these regions.

Arrighi and Hsu (1971) using denaturing/renaturing techniques found that the centromere areas of the no. 3 chromosomes stain deeply with Giemsa and consist mainly of heterochromatin. In the use of proteolytic enzymes (Seabright, 1972) and the ASG technique, (Sumner et al, 1971) as pretreatments, bands were seen near the centromere and on the distal regions of both arms. These regions are the same ones that show positive staining with quinacrine.

The visual identification of this chromosome, therefore, presents no problem. In fluorescence studies, the intense band near the centromere is striking, if present. The nearly symmetrical patterns on both arms, with dull bands in the median regions, are characteristic features of this chromosome.

GROUP 4 - 5(B)

The four chromosomes that constitute Group 4 - 5(B) according to the classification decided on at the Denver and London Conferences can not be identified separately by conventional staining methods alone. On the basis of size and centromere position, the four chromosomes can be distinguished as a group from the other

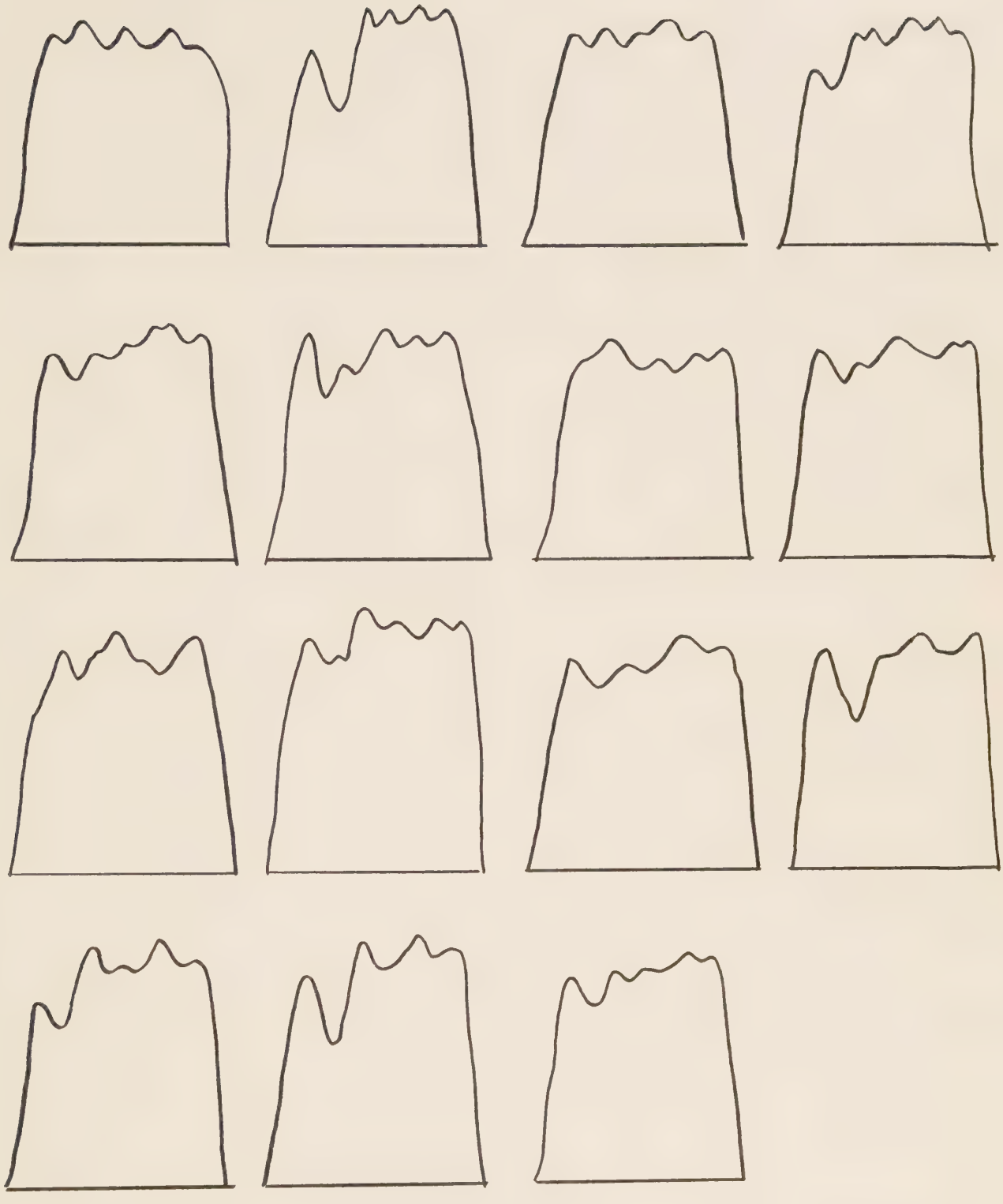

Figure 10 - Wave pattern recordings of 15 fluorescent no. 4 chromosomes.

chromosomes in the karyotype. The chromosomes in the B-group have subterminally placed centromeres and measurement studies reveal they have low centromere indexes.

Studies of the DNA synthesis patterns of chromosomes in this group revealed that in cells labelled near the end of the S period, two chromosomes were labelled over most of their length. The other two chromosomes usually have only the short arms labelled. For routine karyotyping, however, the autoradiographic technique is too tedious and time consuming.

The fluorescent patterns for chromosomes 4 and 5, after staining with quinacrine mustard, are strikingly different. (Figures 4, 10 and 11).

Chromosome number 4.

Chromosome no. 4 can be identified from no. 5 as having an even, bright fluorescence over the long arm: no. 5 chromosome has a long arm with a distal light fluorescensing region.

The short arm of the no. 4 chromosome stains bright proximal to the centromere (p1). The median region stains light (p3) and the distal region is dull fluorescing. The proximal band, p1, was observed in one individual out of 50 studied as being strikingly intense. Manolov et al (1971) states that an intense region is often visible. It seems therefore that this p1 band is polymorphic in its staining characteristics.

Arrighi and Hsu (1971) demonstrated a fairly large heterochromatic centromeric region on one pair of B-group chromosomes. With the ASG technique only one band was observed by Evans et al (1971) in the median region of the short arm.

In fluorescence preparations the long arm of the no. 4 chromosome stains with an overall bright fluorescence. Four evenly spaced, broad bands can be seen on the long arm in good preparations with band q1 proximal to the centromere. This confirms the results of Caspersson et al (1971) and Manolov et al (1971).

The pair of late replicating chromosomes in autoradiographic studies are most probable the no. 4 chromosome. A correlation between late DNA synthesis and bright banding patterns has been observed. (Ganner and Evans 1971). This correlation in the B-group chromosomes has not yet been confirmed by simultaneously performing DNA synthesis and fluorescence studies.

In the Giemsa and ASG techniques, Sumner et al (1971) observed 4 dark staining bands on the long arm.

Chromosome number 5.

Chromosome no.5 can be identified in the karyotype by its fluorescence pattern after staining with quinacrine mustard. (Figures 4 and 11).

The short arm stains very characteristically with a bright median band and dull negative bands in the proximal and distal regions. This corresponds with the bands seen by Manolov et al (1971) and Caspersson et al (1971) in quinacrine stained preparations, and with the positive bands stained with the Giemsa techniques.

In autoradiographic studies, Gianelli (1970) reported that a pair of Group B chromosomes label only on the short arms in cells labelled near the end of the S period. This pair is probably the no. 5 chromosome on the evidence of Ganner and Evans (1971) regarding a correlation between late DNA synthesis and bright fluores-

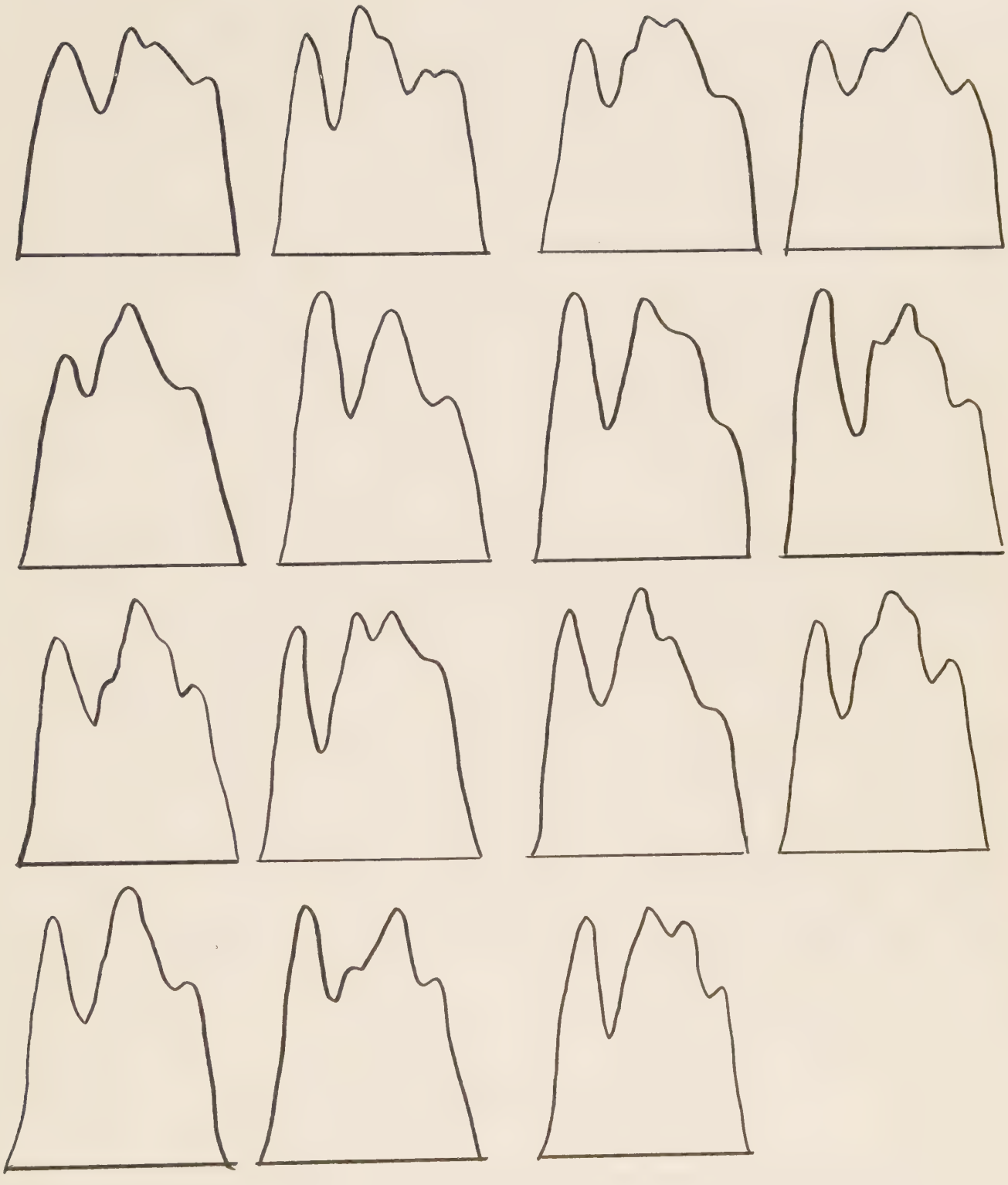

Figure 11 - Wave pattern recordings of 15 fluorescent no. 5 chromosomes.

cence.

On the long arm a broad, bright band, probably a fusion of the bands q3 and q5 described by Manolov et al (1971), was observed in the median region. A characteristic, dull fluorescing region is followed by a light band on the distal region. The distal band q9, which Manolov et al (1971) described as being bright (++), was consistently found to be of relative light intensity on doing fluorometric measurements of the wave pattern. Bands q3, q5 and q9, recorded photo-electrically, were not found to be of the same intensity as described by Manolov et al (1971). Bands q3 and q5 gave higher intensity measurements than the band q9. Manolov et al (1971) described a total of 5 positive bands on the long arm.

In Giemsa preparations Evans et al (1971) found a characteristic, broad band in the median region of the long arm with a small band on each side.

By doing fluorescent studies of the 4 - 5(B) group chromosomes the individual chromosomes can easily be identified. The main differences between these two chromosomes are the even, bright flourescence of the no. 4 as compared to the no. 5, which has characteristic negative, dull centromeric region and a bright and a light band on the long arm.

GROUP 6 - 12 AND X(C)

The chromosomes in this group are difficult to identify with certainty from conventional stained preparations since the chromosomes are all of approximately the same length and have rather similar centromere indexes. The longer chromosomes numbers 6, 8 and X, can be separated as a group from the smaller chromosomes nos.

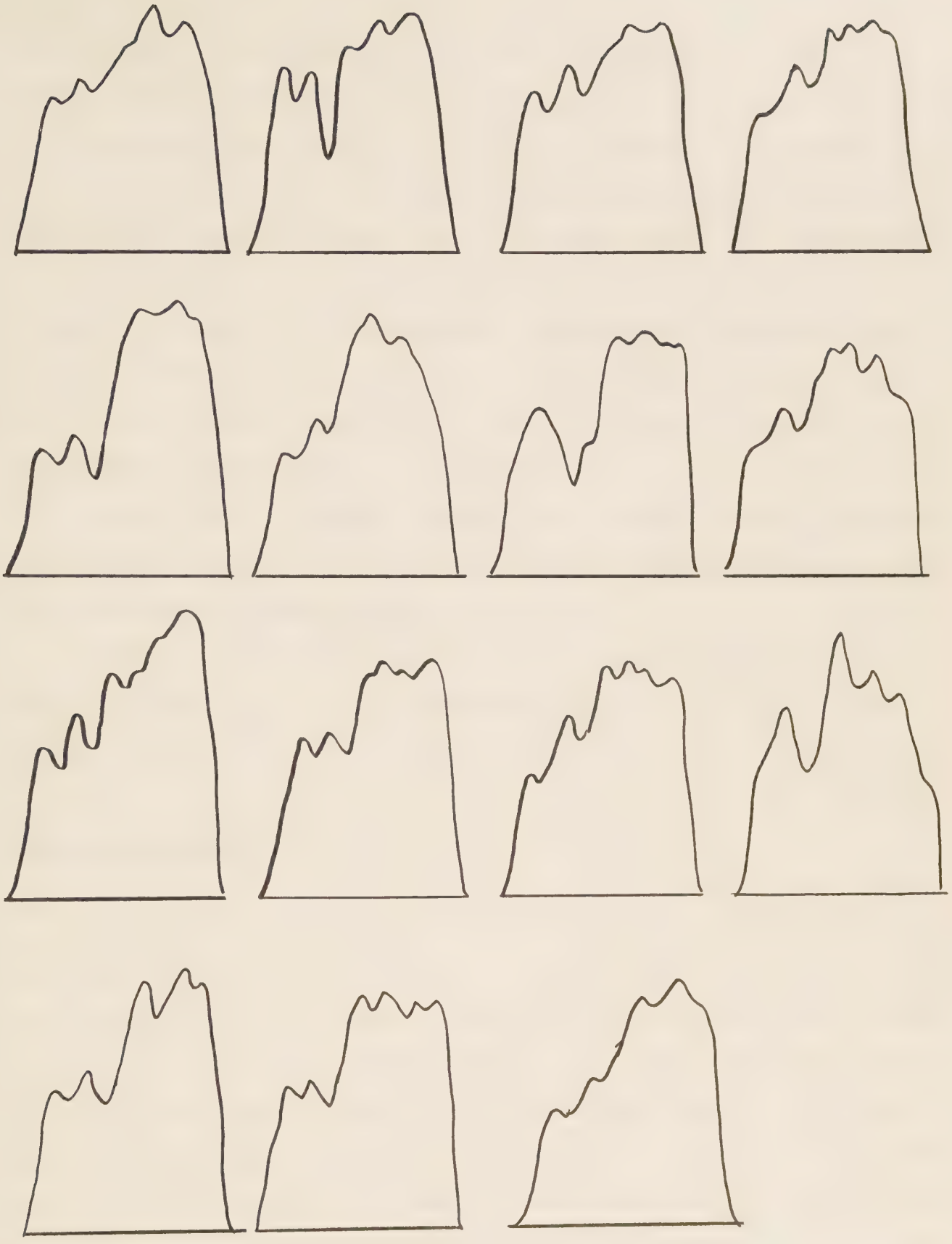

Figure 12 - Wave pattern recordings of 15 fluorescent no. 6 chromosomes.

10, 11 and 12. The no. 9 chromosome often reveals a secondary constriction on the long arm close to the centromere. Within these groups, however it is difficult to separate the chromosomes with certainty. Thus clinical syndromes associated with structural abnormalities of the C-group autosomes have not been defined.

One of the X chromosomes in the C-group can be identified as a very late replicating chromosome by means of autoradiographic studies. The other chromosomes in the C-group are too numerous and morphologically similar to assign different labelling patterns to specific chromosomes. (Gianelli, 1970).

In well stained quinacrine mustard preparations the C-group chromosomes can be arranged individually in their pairs in a normal karyotype of human chromosomes.

Figure 5 shows a series of C-group chromosomes stained with quinacrine mustard. The average wave pattern recorded for the chromosomes in each of the groups are given.

Chromosome number 6.

The short arm of this chromosome is characterized by a dark negative band in the median region, designated as p2. The proximal positive band is brightly fluorescent, while the broad distal positive band has light fluorescence. As demonstrated in figure 12, the distal band on the short arm (p3) was seen in most preparations to be lightly fluorescent and measured photo-electrically had a lower intensity than band p1, more proximal to the centromere. In their first publication Caspersson et al (1970a) discribed the p3 band as being of higher intensity.

The ASG preparations of Sumner et al (1971), and Evans et al

(1971), as well as the proteolytic enzyme banding patterns of Seabright (1972), show a broad negative staining median region surrounded by dark stained bands.

The long arm of this chromosome staines rather featurelessly, ranging from bright to light fluorescence, while the proximal region gives, on the average, the highest intensity measurements. (Figure 5). Manolov et al (1971) found 4 positive bands on the long arm and described q1 as bright in intensity, and the others light. Caspersson et al (1971) described the long arm as evenly fluorescent with faint bands discernible.

The 4 bands on the long arm have also been identified on Giemsa stained preparations with various pretreatments. (Evans et al 1971, Sumner et al 1971, Seabright, 1972).

Chromosome no. 6 can with certainty be identified visually after staining with quinacrine mustard by virtue of the characteristic banding of the short and long arm. Striking features are the dull negative band in the median region of the short arm, and the even featureless fluorescing of the long arm.

Chromosome number 7.

Chromosome no. 7 has a very striking fluorescence pattern with 3 characteristic brightly fluorescing regions. Identification can be made with certainty from a well-stained metaphase, using quinacrine mustard. (Figures 5 and 13).

The short arm has a light fluorescent band p1, proximal to the centromere, followed by a narrow dull band p2, and a broad, bright distal band p3. The centromere region has a dull fluorescence. The long arm is characterized by two bright median bands separated

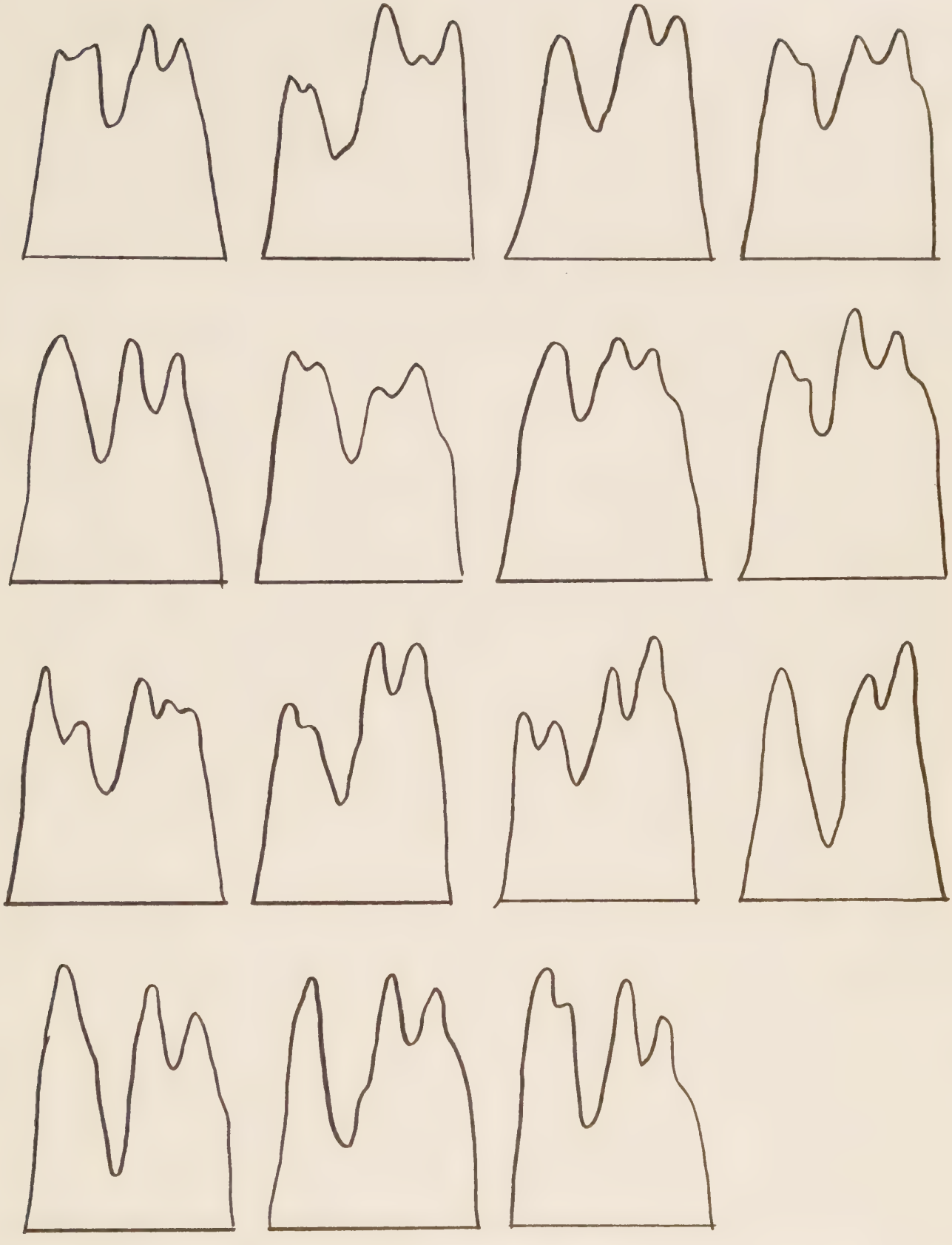

Figure 13 - Wave pattern recordings of 15 fluorescent no. 7 chromosomes.

by a dull region, and a light band in the distal region. The three bright bands, p3, q3 and q5, are very characteristic of this chromosomes. Manolov et al (1971), in their study of longer chromosomes, have recognized a small positive band on the long arm, very proximal to the centromere, which they designate as q1. The three main bands were also seen by Caspersson et al (1971) and Evans et al (1971) using quinacrine mustard and Giemsa stained preparations.

Chromosome number 8.

Chromosome no. 8 stains rather poorly with quinacrine mustard. The positive bands are all light in intensity. On the average, the short arm fluoresces with a lower intensity than the long arm. In the wave pattern recordings intensity measurements are standardized within a chromosome so that only intensities between bands on the same chromosome can be compared. Intensity differences between chromosomes in the same cell are not taken into account because of technical difficulties. Measured subjectively however, chromosome no. 8 stained with an overall intensity that was lighter than the other chromosomes in the same group. Chromosome no. 8 is visually often best identified by a process of elimination.

The short arm stains with a light overall fluorescence. Manolov et al (1971) described two lightly fluorescing broad bands separated by a narrow dull band. The long arm has a relatively higher fluorescence compared to the short arm, and in good preparations two light bands can be recognized, with a dull distal region. This dull distal region gives the chromosome a shorter appearance because of the difficulty in defining the dark telomere region. On the long arms of elongated chromosomes Manolov et al

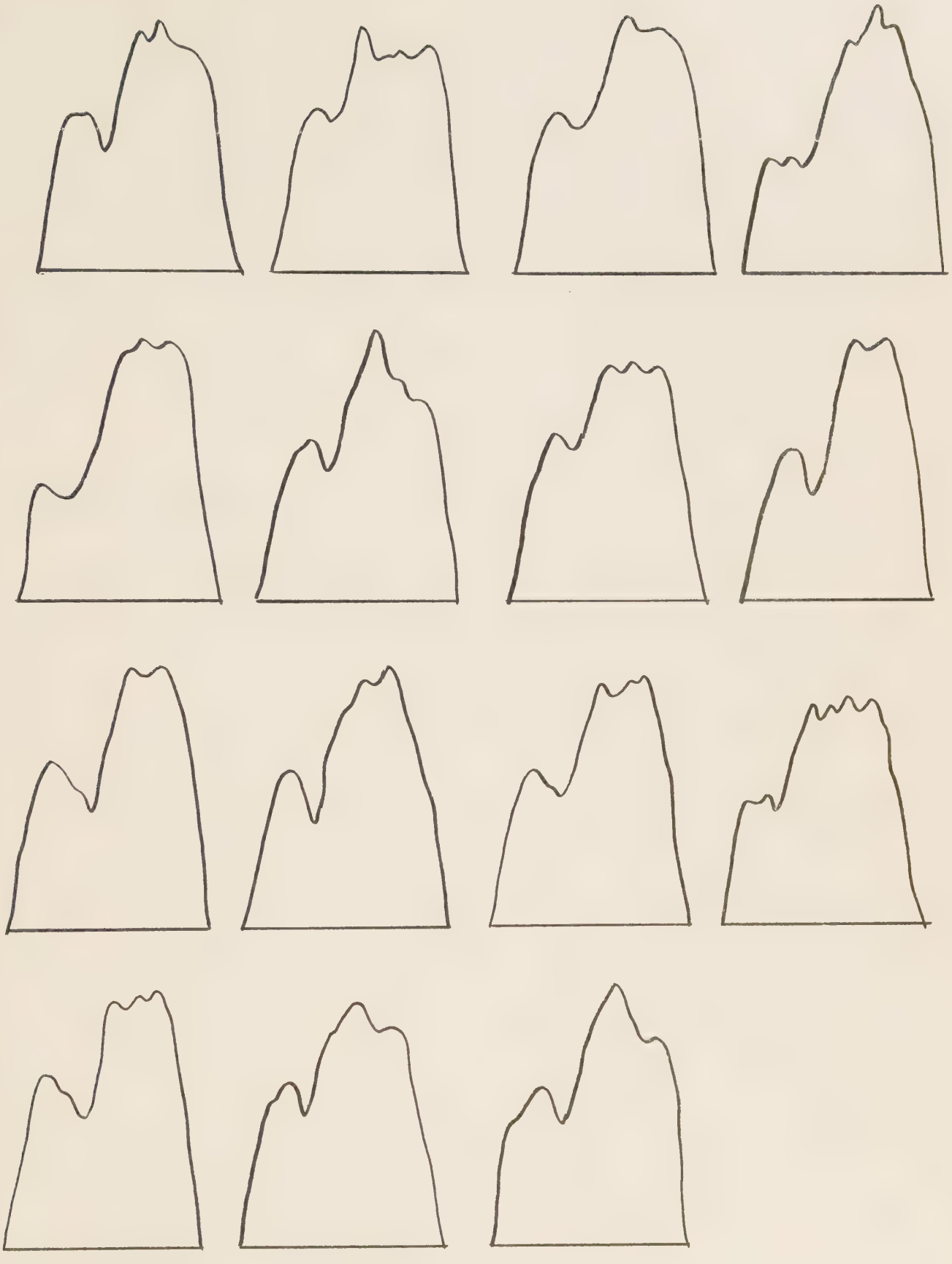

Figure 14 - Wave pattern recordings of 15 fluorescent no. 8 chromosomes.

(1971) described 3 light positive bands.

The photo-electric wave patterns of various no. 8 chromosomes given in figure 14, show a pattern which have characteristic light intensity readings on the short arm when compared to the long arm.

In a comparison between Giemsa and fluorescent stained regions, Evans et al (1971) found that this chromosome exhibited few features with either technique. On very good cells, he found two narrow bands in the short arm and two wider bands on the long arm.

Chromosome number 9.

The fluorescent pattern of chromosome no. 9 is peculiar to it mainly because of the secondary constriction region on the long arm which does not stain in quinacrine mustard preparations. In some preparations it gives the appearance of two separate chromatin bodies. (Figure 5).

The short arm fluoresces with a bright intensity on the distal band and a lighter proximal region. The centromere region, as well as a broad proximal region of the long arm is dark, followed by two bright fluorescent bands in the median and distal regions of the long arm. Manolov et al (1971) described the short arm as having two positive bands, a light p1 and bright p3, separated by a very narrow, dull fluorescent region. The bands found on the long arms in this study correspond with the findings of Caspersson et al (1971) and Manolov et al (1971).

In denaturing/renaturing studies Arrighi and Hsu (1971) described one pair of chromosomes in the C-group as having a Giemsa-positive region which is polymorphic in size and consist largely of heterochromatin. This was believed to be the no. 9 chromosome

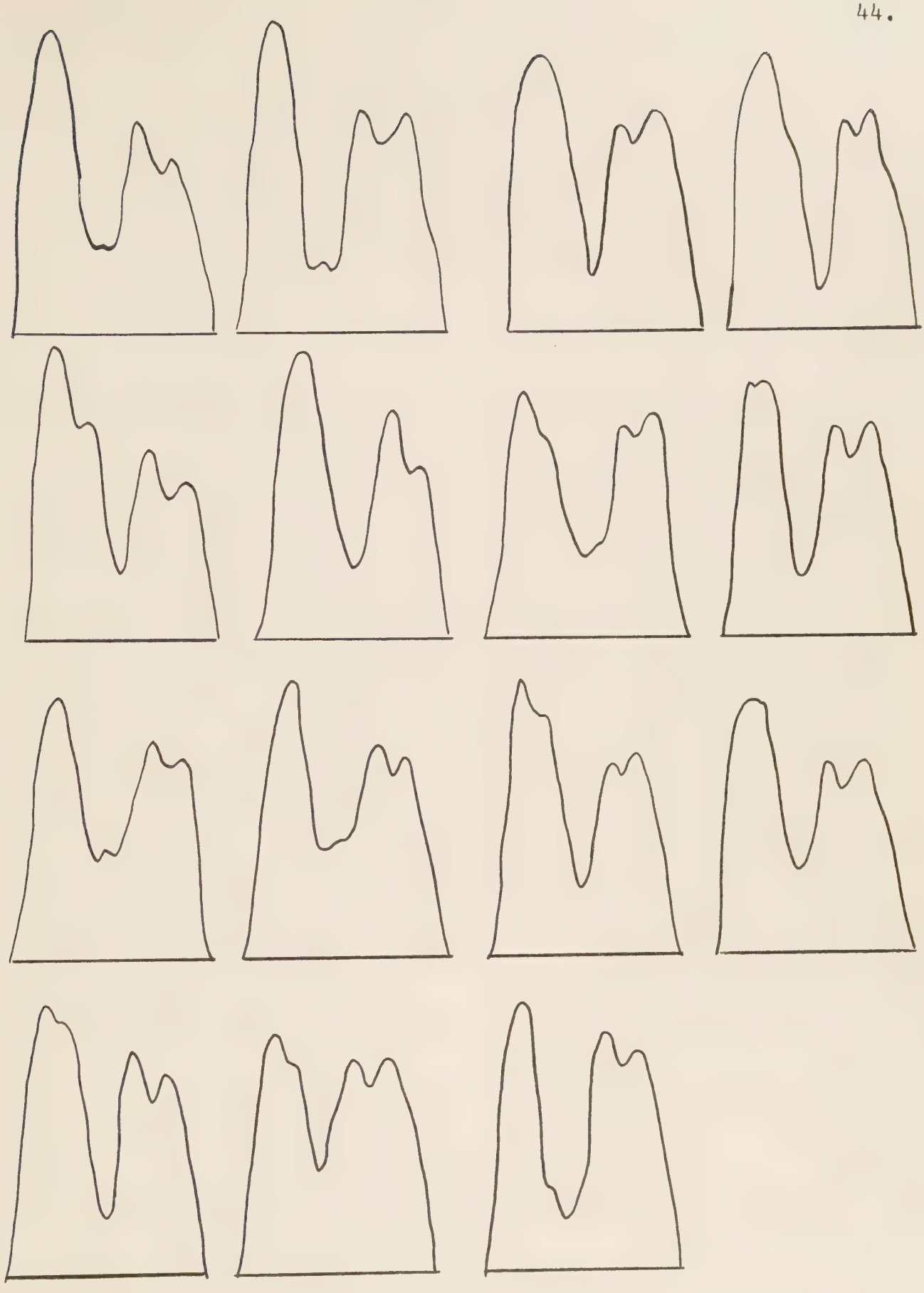

Figure 15 - Wave pattern recordings of 15 fluorescent no. 9 chromosomes.

with the secondary constriction area.

The ASG techniques give similar results to the fluorescing technique in showing negative staining in the secondary constriction regions, with one band on the short arm and two on the long arm. (Evans et al 1971).

Chromosome number 10.

Chromosome no. 10 is one of the smaller chromosomes in the C-group with a subterminally placed centromere. In fluorescence studies this chromosome is mainly characterized by a brightly fluorescent band located proximally on the long arm.

The short arm of this chromosome stains with a light, evenly fluorescing band in the median region, surrounded by dull fluorescent regions. The long arm has a characteristic bright band q1 proximal to the centromere, followed by light fluorescence on the rest of the arm. Manolov et al (1971) described another two lightly staining bands, q3 and q5, on long chromosomes. Evans et al (1971) described the banding of this chromosome after staining with Giemsa in the ASG technique, as having a band on the short arm and 3 bands on the long arm, the more proximal one being the most positive.

This chromosome can only be identified in well-stained preparations. In cells that do not show particularly good differentiation however, the no. 10 chromosome is not easy to recognize.

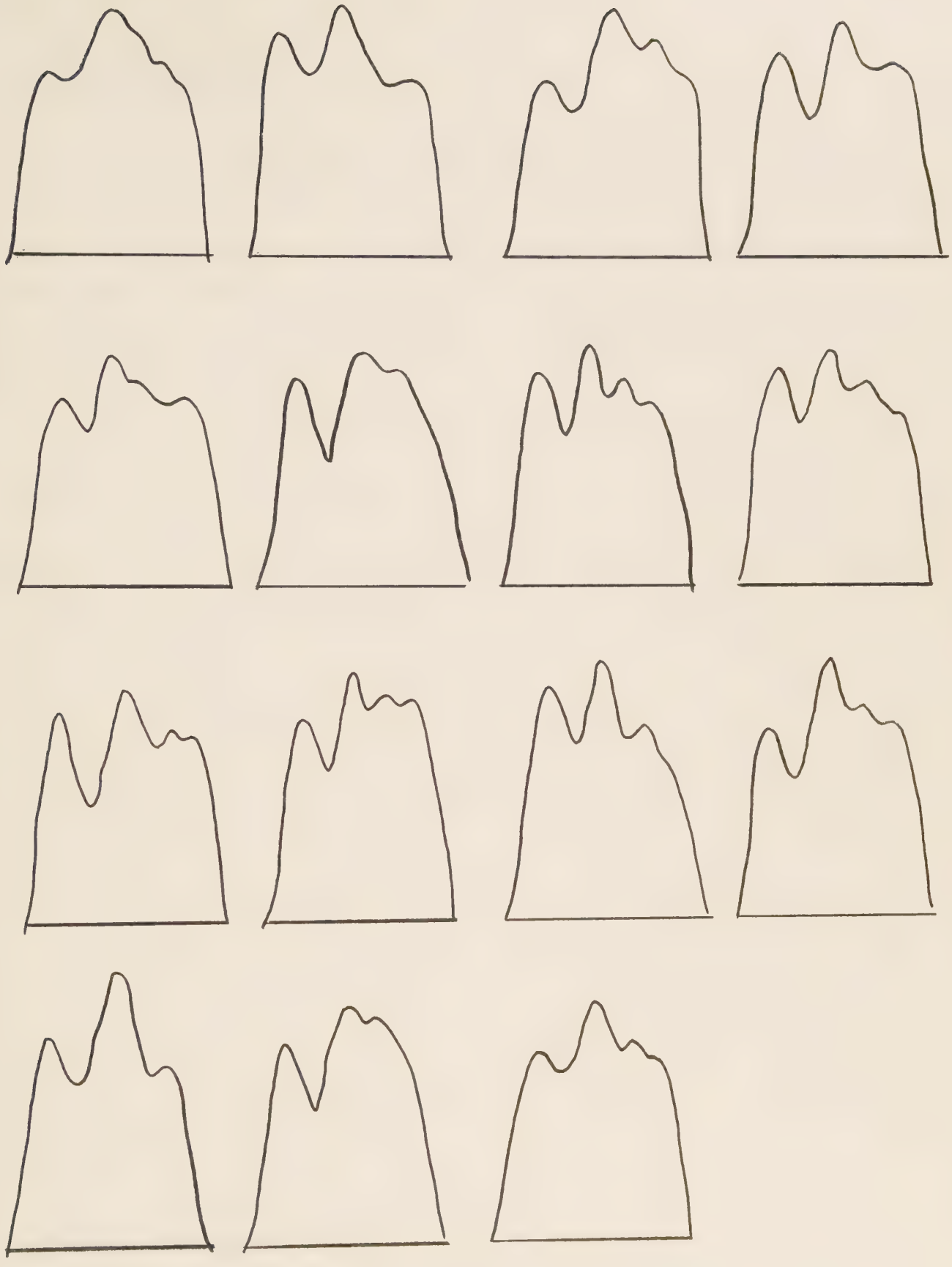

Figure 16 - Wave pattern recordings of 15 fluorescent no. 10 chromosomes.

Chromosome number 11.

In fluorescence studies chromosome no. 11 is one that exhibits particularly clear bands. Two main features are characteristic of this chromosome, and enable visual identification to be made easily, namely, a broad band on both the short and long arms with a small, narrow positive band proximal to the centromere on the long arm.

The photo-electric recordings of this chromosome reveal a nearly symmetrical pattern (figure 17). A bright band is seen on the short arm with a dull region proximal to the centromere. Manolov et al (1971) in studying long chromosomes, have observed two very close positive bands on the median region of the short arm and a narrow band on the distal region. On staining the long arms exhibit a characteristic, narrow positive band proximal to the centromere followed by a dull region and a broad bright median band. Manolov et al described two bands with a very narrow negative region in between on the long arm, while Caspersson et al (1971) observed a pattern similar to that found in this study.

In Giemsa (ASG) stained preparations, as well as when using proteolytic pretreatments of chromosomes of medium length, only one positive band was seen on the short arm: while 2 bands, one broad and one narrow, were seen on the long arm. (Evans et al 1971, Seabright 1972).

Chromosome number 12.

Chromosome no. 12 is the one chromosome with the shortest short arms in the C-group. When stained with quinacrine mustard

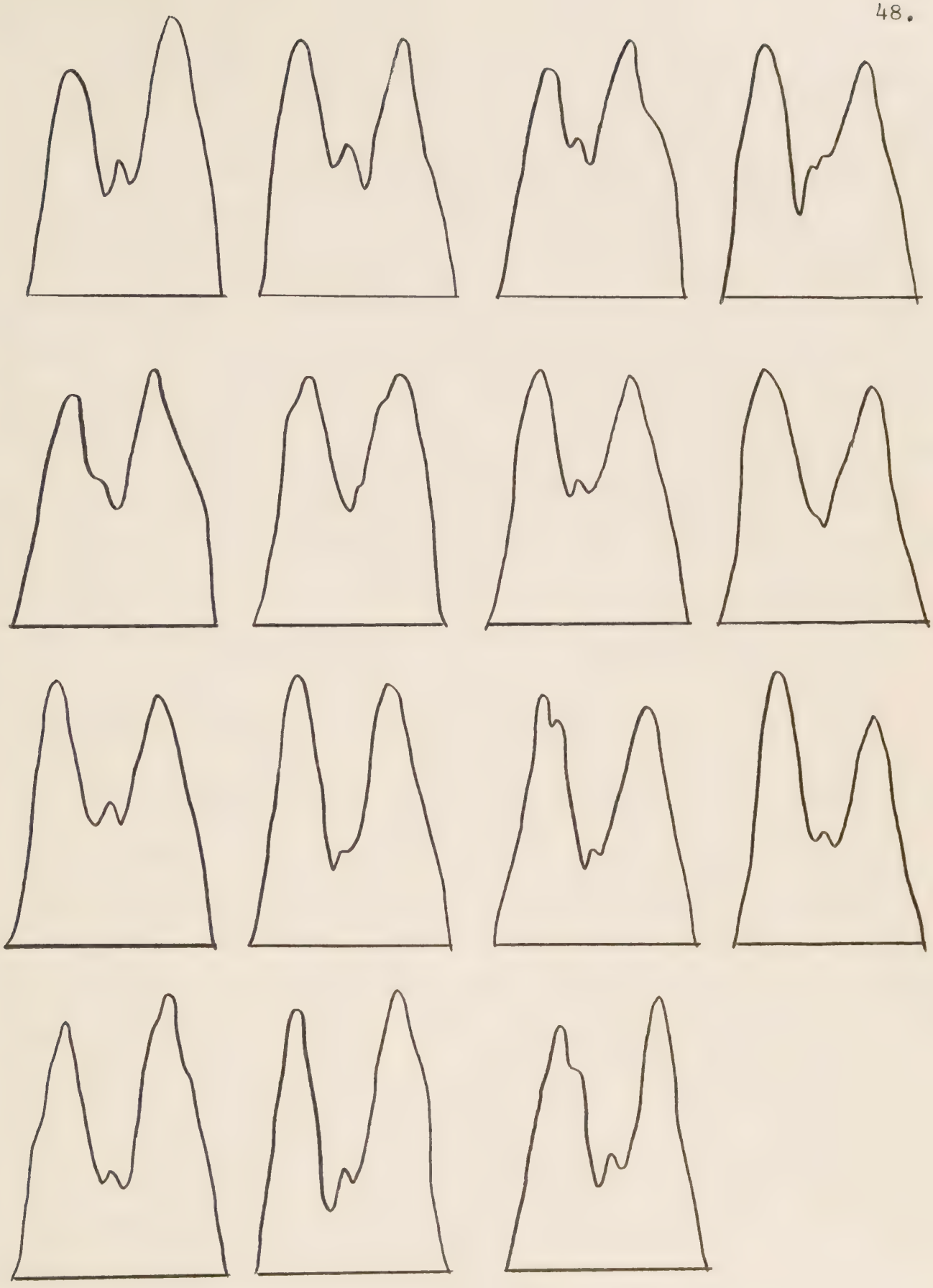

Figure 17 - Wave pattern recordings of 15 fluorescent no. 11 chromosomes.

this chromosomes is easily identified for karyotyping. (Figures 5 and 18).

On staining, the short arm has a light median band surrounded by dull fluorescence in the distal and centromere regions. The long arm has a narrow band, q1, very proximal to the centromere, next to it is a negative region and then a broad, bright positive band in the median region. The distal region of the long arm is characterized by a dull fluorescence. This pattern confirms the findings of Evans et al (1971) and Caspersson et al (1971). Manolov et al (1971) described the long arm as consisting of 3 bands rather than two. The bands q3 and q5 were described as light and bright in fluorescence respectively. In more contracted chromosomes these bands fuse to give the appearance of one broad band.

The X-chromosome.

The X-chromosomes of the female, unlike the other chromosomes in the C-group, vary in length between two homologues, in the same cell. In most cells, the one X chromosome appears to be more contracted than the other. In autoradiographic studies, one of the X chromosomes is found to replicate its DNA late in the S period. Ganner and Evans (1971), in their study of the relationship between patterns of DNA replication and quinacrine fluorescence found that the chromosome regions showing bright fluorescence were also late replicating. Exceptions to this generalization included, amongst other smaller regions, the late labelling X chromosome in cells of female origin. Both X chromosomes in the female were found in quinacrine stained preparations to stain with the same fluorescence pattern.

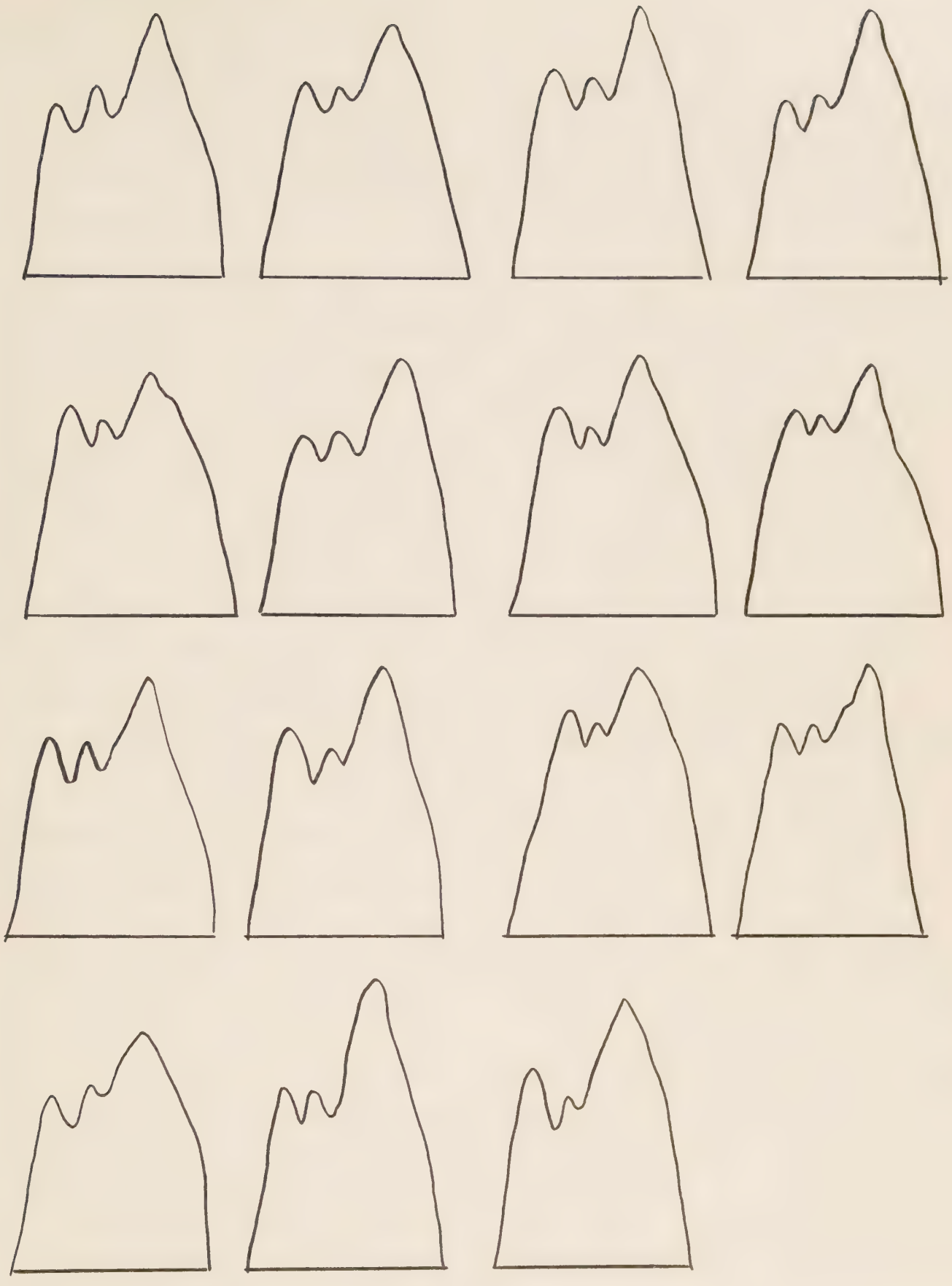

Figure 18 - Wave pattern recordings of 15 fluorescent no. 12 chromosomes.

In fluorescence studies, the X chromosome does not show up marked contrasts between bands. The photo-electric recordings of the banding patterns in figure 19 show a regular curve of brightly fluorescent measurements on both arms and a negative fluorescing centromere region. Photo-electric measurements show that the bright fluorescence on the short and long arms are of equal intensity. The short arm fluoresces with a bright band in the median region. The long arms have a bright band in the median region and gradually decline in intensity towards the telomeric region. Manolov et al (1971) described 3 bands on the long arm, with the distal region divided into two lightly fluorescent areas.

The fluorescence pattern of the no. 10 chromosome and that of the X chromosome appear quite similar. The X chromosome however, has a higher centromere index and in pattern recordings shows a much broader negatively fluorescing region near the centromere than the no. 10. The bright band q1, on the long arm of no. 10 chromosome appears more intensely fluorescent compared to the other bands on the same chromosome.

Evans et al (1971) observed one well-marked band on both the short and the long arm and found that the other bands were less well marked.

Although the X chromosome does not exhibit marked characteristics when stained with quinacrine mustard, it can, in well-stained preparations, be karyotyped on its banding pattern. Careful examination of chromosomes nos. 8, 10 and X reveal 3 distinct types of chromosomes and by a process of elimination they can confidently be karyotyped.

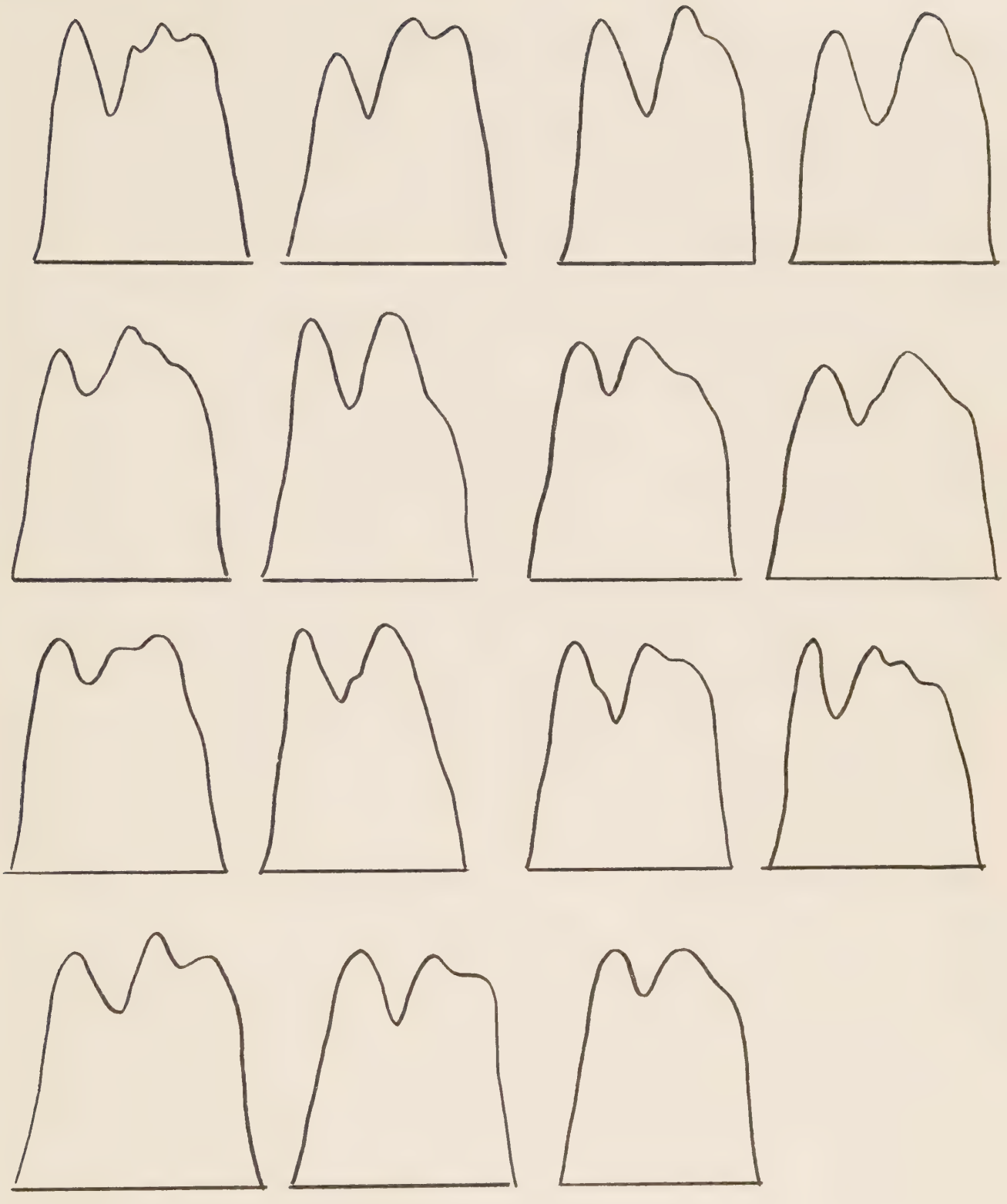

Figure 19 - Wave pattern recordings of 15 fluorescent X chromosomes.

GROUP 13 - 15(D)

The chromosomes of the 13 - 15(D) group of the normal human karyotype are medium-sized with nearly terminal centromeres. These acrocentric chromosomes can easily be identified as a group:- chromosomes of other groups of nearly similar lengths are submetacentric. In conventionally stained metaphases, these chromosomes cannot be identified individually. The chromosomes in this group are polymorphic for the presence and size of satellites on the short arms. The presence of these satellites, influences the measurement studies of these chromosomes. In identification studies of D-group chromosomes by autoradiography, Gianelli and Howlett (1966) have, for this reason, measuredonly the long arms. Penrose (1964), in summarizing the data presented at the Denver and London Conferences on acrocentric chromosomes, concludes that logically it appears possible to group them only according to the measurements of their long arms.

The allocation of numbers to specific pairs of chromosomes in this group was based on the results of measurements and autoradiographic studies. (German, 1964, Schmid, 1963, Gianelli and Howlett 1966 and others). The longest pair of D-group chromosomes (no. 13) are the last to complete DNA synthesis and are heavily labelled on the distal part of the long arm. The pair of chromosomes of intermediate length (no.14) are second to complete DNA synthesis and have a region of late DNA synthesis in the short arm and in the proximal region of the long arm. The shortest chromosomes (no. 15) are the first to complete DNA synthesis.

In fluorescence studies on the D-group chromosomes, the banding patterns of the long arms alone were used for identification.

The variations in length and size, and the presence of satellites on the short arms of these chromosomes, made it difficult to standardize these regions photo-electrically. A great difference in fluorescent intensity occured between satellites of homologous chromosomes and also between satellites of different chromosomes in this group. When comparing the fluorescence patterns of the long arms of the six D-group chromosomes in the karyotype, three distinct types of patterns were recognized (figure 6).

Chromosome number 13.

The chromosome no. 13 exhibits intense fluorescence in the short arm in 48% of the individuals studied. In 40% of individuals this region of intense fluorescence was seen only on one of the pair of no. 13 chromosomes. In the remaining 8% both the no. 13 chromosomes stained with intense fluorescence in a small region proximal to the centromere on the short arm. A band of similar intensity was not seen on any of the other chromosomes in this group.

The long arm of chromosome no. 13 stains very characteristically with quinacrine mustard and can be identified on the metaphase of human chromosomes. (Figures 6 and 20). The proximal region has a dull fluorescence, while the median and distal regions fluoresce with broad bright bands. In well-stained preparations two bands could be seen in the median region. Manolov et al (1971) described another two light bands close to the broad bands, one on either side. These regions of dull proximal fluorescence and bright distal fluorescence are peculiar to the no. 13 chromosome, and enable visual differentiation from the other two pairs to be

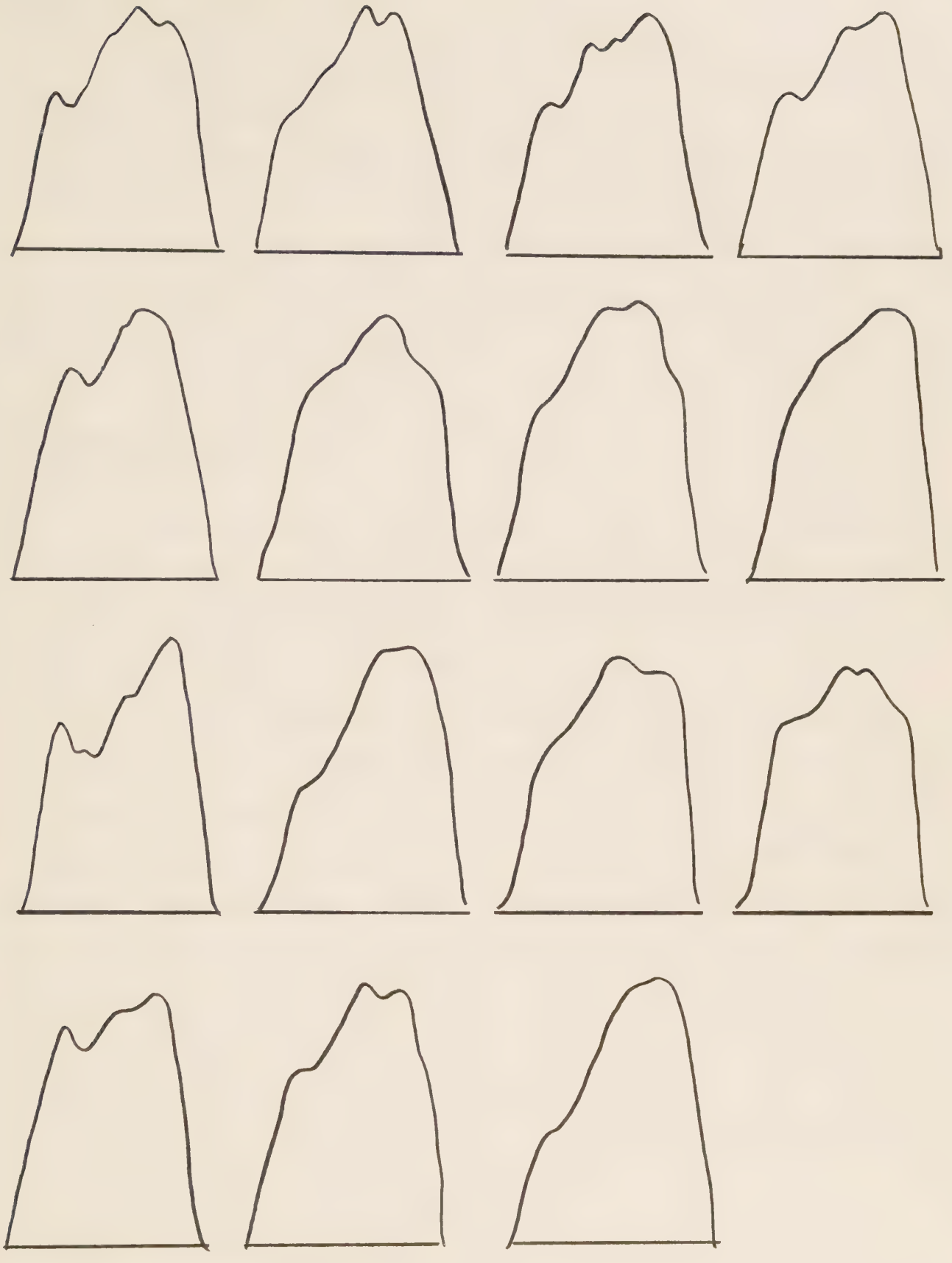

Figure 20 - Wave pattern recordings of 15 fluorescent no. 13 chromosomes.

made. The wave pattern recordings of several no. 13 chromosomes are shown in figure 20.

Autoradiographic studies show that the median and distal parts of this chromosome complete DNA synthesis late. (Gianelli, 1970).

In Giemsa preparations the long arms exhibit two distal bands and a pale band proximal to these, with a polymorphic band on the short arm. (Evans et al 1971).

Chromosome number 14.

Chromosomes nos. 14 and 15 have rather similar regions of positive fluorescence. On metaphases where 5 to 6 D-group chromosomes are present it is easy to differentiate both the no. 14 chromosomes from the no. 15 chromosomes.

The no. 14 chromosome as can be observed in figure 6 and the photo-electric recordings in figure 21, stains with a bright fluorescent band proximal to the centromere, corresponding to bands q1 and q3 of Manolov et al (1971). A second bright band lower in intensity than the first, is seen in the distal region. Chromosome 14 can be distinguished by the presence of this bright distal band, since the no. 15 chromosome has a dull, distal region.

In autoradiographic studies chromosomes no. 14 was found to complete DNA synthesis relatively late in regions near the centromere. Giemsa preparations show a broad band proximally and a distal narrow band. (Evans et al, 1971).

Chromosome number 15.

The characteristic fluorescent feature of chromosome no. 15

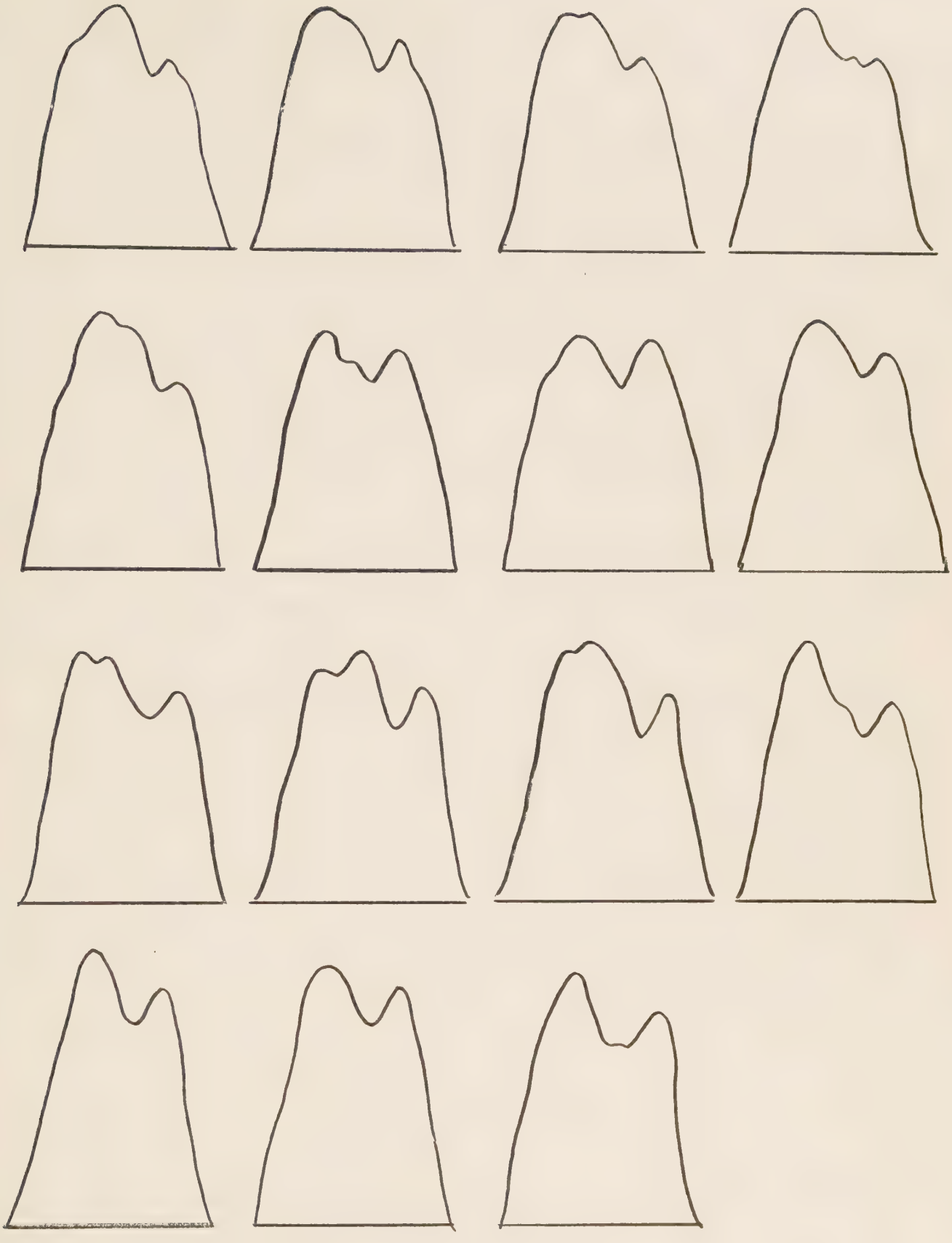

Figure 21 - Wave pattern recordings of 15 fluorescent no. 14 chromosomes.

are the overall light fluorescence and a dull distal region on the long arm.

The chromosome stains with a dull to light fluorescence in the proximal region with the highest intensity being recorded in the median region. The distal region ends with a dull fluorescent band. Manolov et al (1971) described the long arm as having three light fluorescent bands. The proximal band q1, a median band q2, and a distal band q3, all had the same intensity, measured subjectively. In present study however, the distal region was found to stain with a much duller intensity than the rest of the long arm.

This shortest D-group chromosome completes DNA synthesis early and is therefore lightly labelled at the end of the S period of the cell cycle. (Gianelli, 1970).

In Giemsa banding studies of the no. 15 chromosome using the ASG technique Sumner et al (1971), observed less overall staining compared to the other D-group chromosomes.

Although the D-group chromosomes could be identified by autoradiographic studies of the replication patterns of DNA, the fluorescence techniques present an easier and less time consuming method of identifying the 3 pairs of chromosomes for karyotype analysis.

GROUP 16 - 18(E)

In the 16-18(E) group, chromosome no. 16 can be identified without referring to any special staining or other techniques. This submetacentric chromosome has the highest centromere index in the group and can be identified by studies of length and centro-

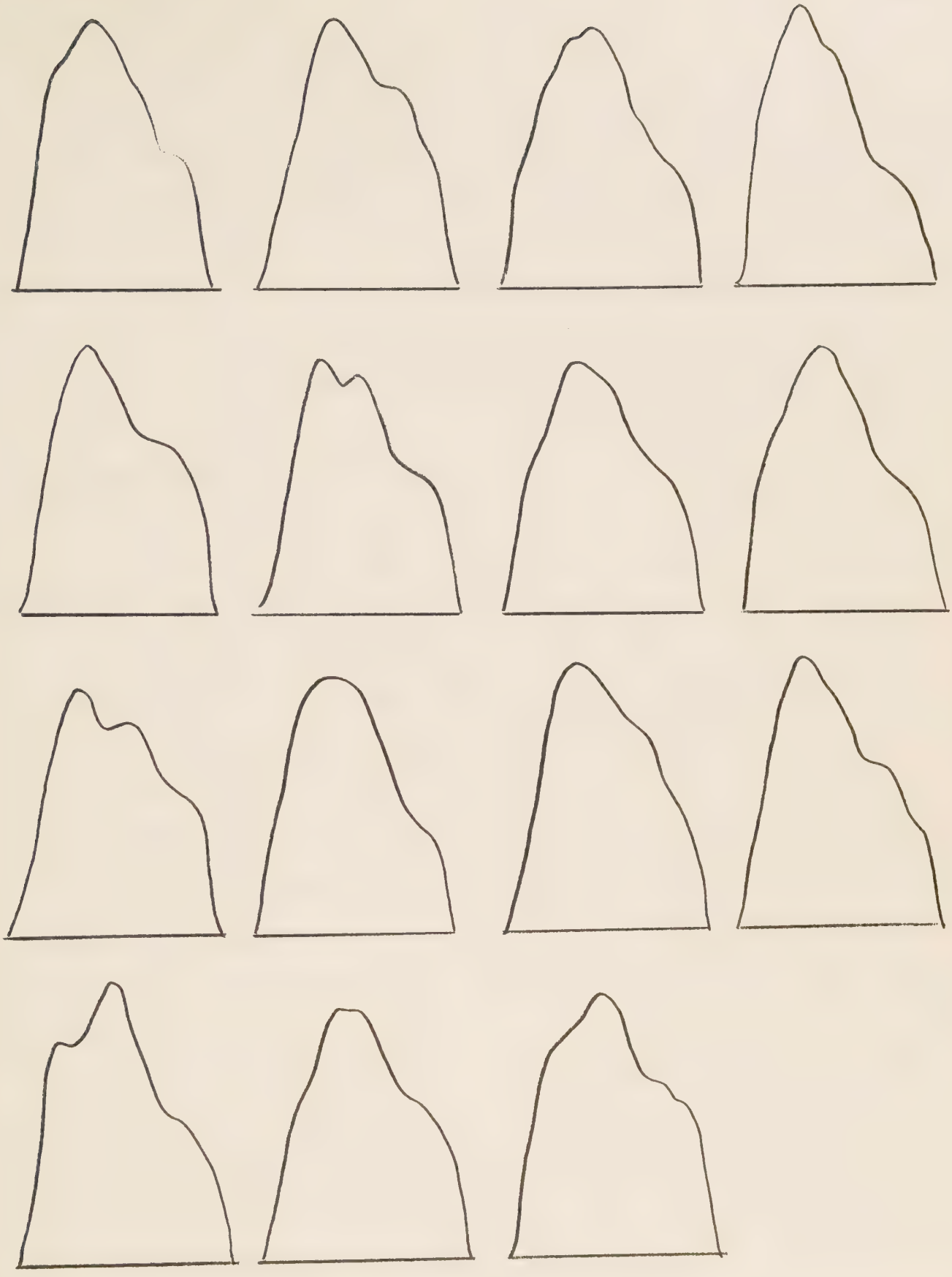

Figure 22 - Wave pattern recordings of 15 fluorescent no. 15 chromosomes.

mere position. A secondary constriction was noted in the proximal region of the long arm. Chromosomes 17 and 18, however show rather similar morphological features when stained with conventional methods. The shorter pair of the two chromosomes which has the more terminally placed centromere, is usually identified as no. 18. Studies of their DNA replication patterns revealed two of the latter chromosomes to be early replicating and two late replicating.

Chromosome number 16.

The results of the quinacrine mustard staining of the no. 16 chromosome are given in figure 6 and the wave pattern recordings of a series of no. 16 chromosomes in figure 23.

Chromosome no. 16 stains with a light fluorescent band on the short arm. The proximal region of the long arm often appears dark, and the length of this region depends on the size of the secondary constriction. The distal part of the long arm fluoresces as a broad bright band. Manolov et al (1971) described two bright bands on the long arm with a very narrow, dull band in between. In contracted chromosomes these two bands probably fuse and appear as one.

On autoradiographic studies the secondary constriction area appears to be late synthesizing. Evans et al (1971) observed an interesting difference between Giemsa and fluorescent staining:- The region of secondary constriction on the long arm proximal to the centromere which is negative in fluorescence studies, stains heavily with Giemsa in the ASG technique.

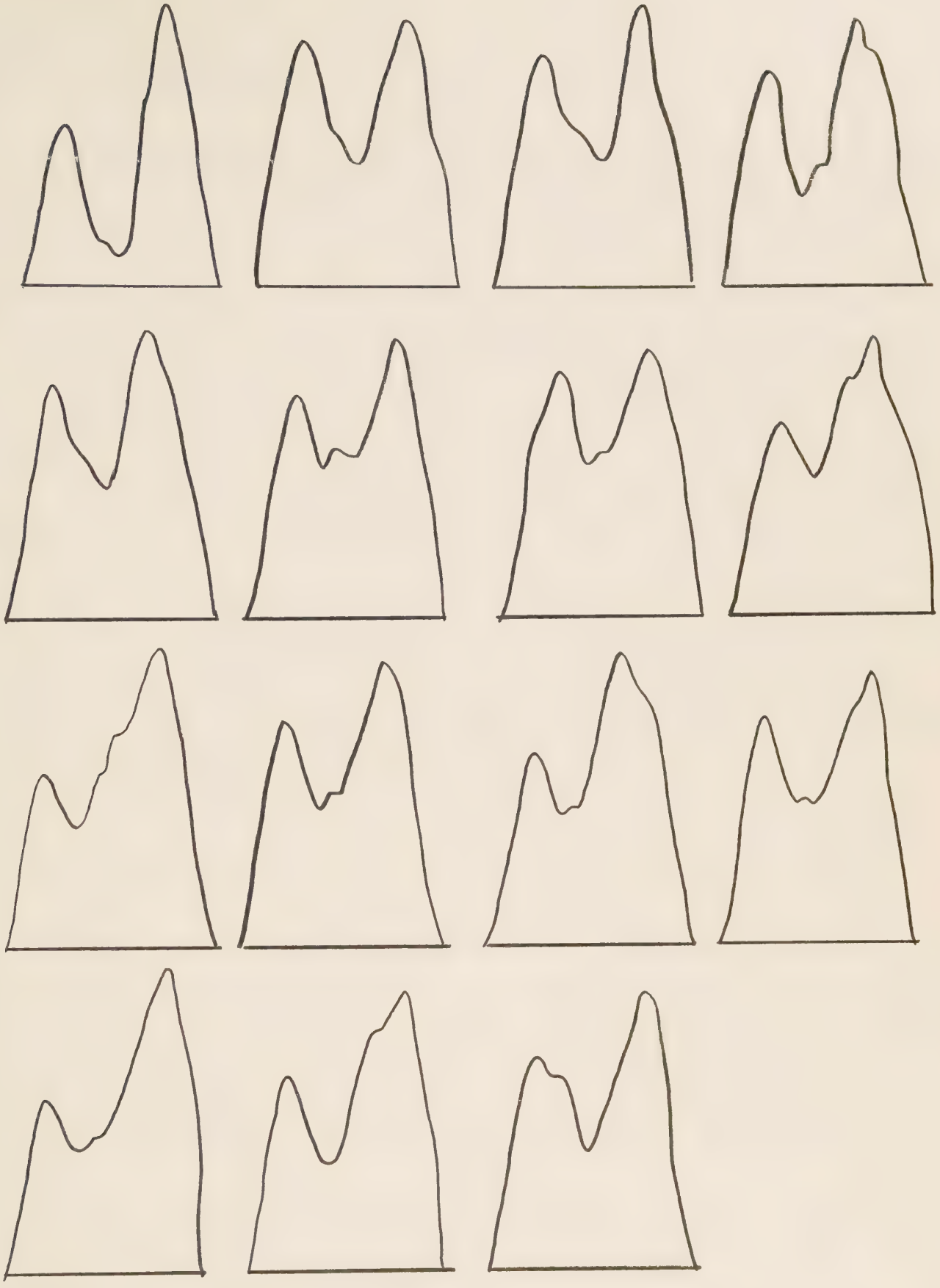

Figure 23 - Wave pattern recordings of 15 fluorescent no. 16 chromosomes.

Chromosome number 17.

When staining with quinacrine mustard chromosome 17 exhibits characteristic fluorometric patterns which differ distinctly from no. 18 (figure 6). The short arm fluoresces with overall light intensity and the long arm with two bands. The proximal band is narrow and light, while a bright broad band is seen in the median and distal regions. This pattern was also described by Caspersson et al (1971).

In the preparations studied by Manolov et al (1971) two bright bands separated by a very narrow dull region were seen on the long arm.

Chromosome no. 17 was found to be early DNA replicating, compared to chromosome no. 18. The Giemsa-staining method revealed one pale band on the short arm and two positive bands on the long arm. (Evans et al 1971).

Chromosome number 18.

As can be seen in figure 6 and the pattern recordings in figure 25 the chromosome no. 18, when stained with quinacrine mustard, fluoresce with light intensity on the short arms, but the two bands on the long arms are bright and of equal intensity. This confirms the studies of Caspersson et al (1971) and Manolov et al (1971).

The no. 18 chromosomes labelled during the end of the S period, appear to be late replicating. (Gianelli, 1970, Ganner and Evans, (1971). In the Giemsa banding preparations Evans et al (1971) observed one band in the short arm and two on the long arm.

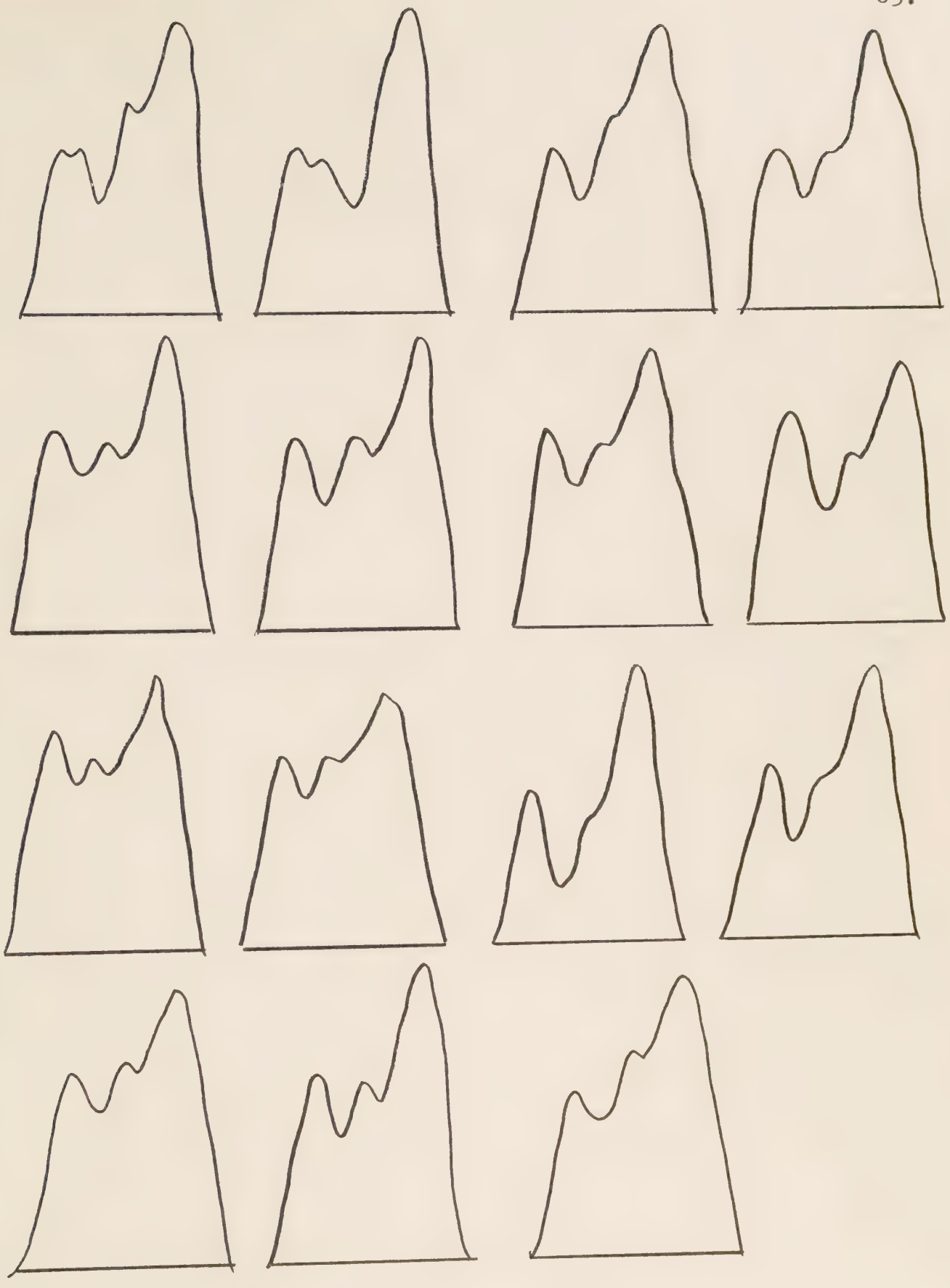

Figure 24 - Wave pattern recordings of 15 fluorescent no. 17 chromosomes.

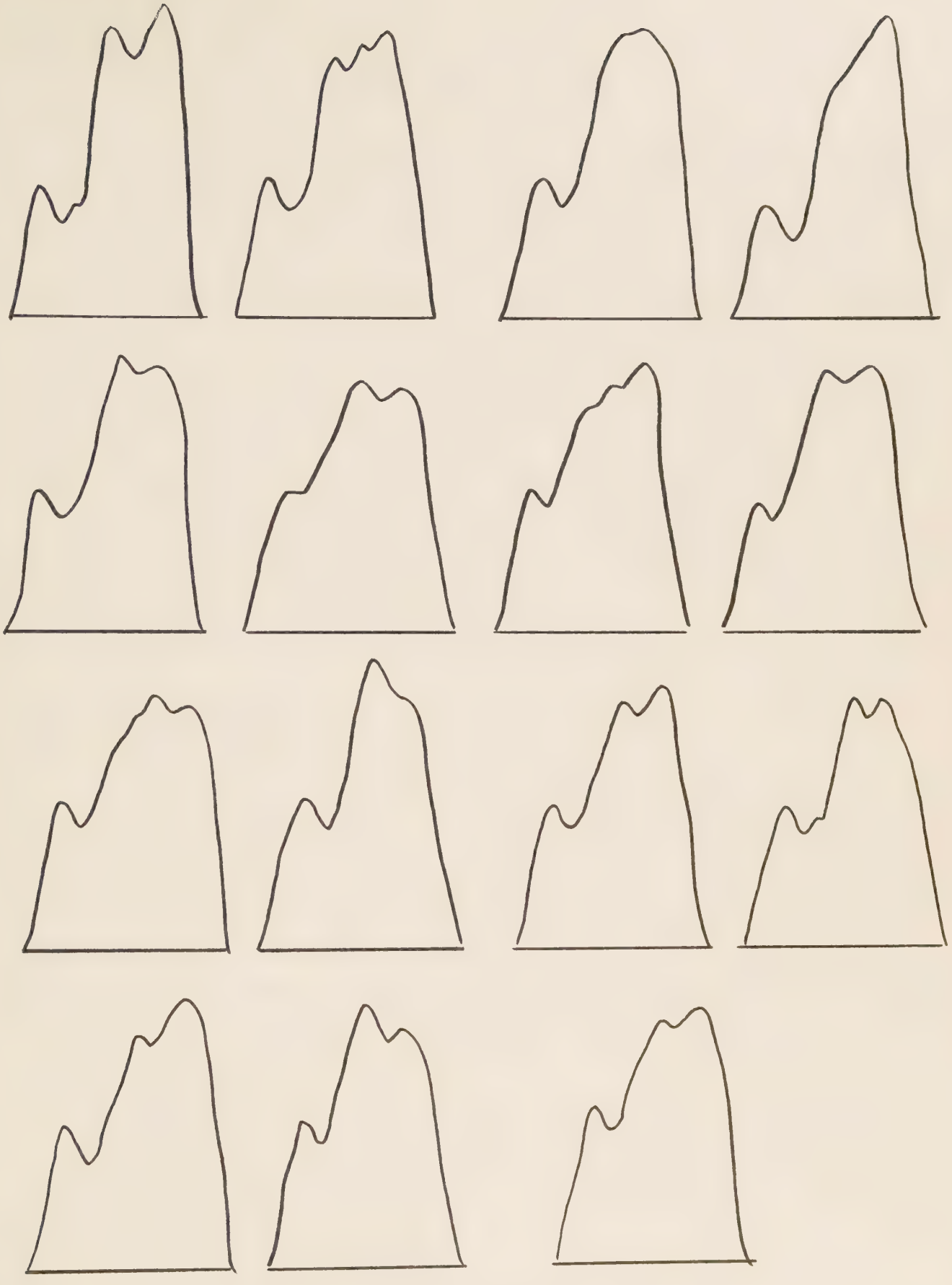

Figure 25 - Wave pattern recordings of 15 fluorescent no. 18 chromosomes.

GROUP 19 - 20(F)

It is impossible to classify the small metacentric chromosomes of the F-group into two distinct types either on their morphological appearance or DNA synthesis investigation. The four metacentric chromosomes can, however, be identified as a group. In DNA replication studies both pairs complete synthesis early.

Studying the fluorescent patterns of these small chromosomes yields few details, but on intensity measurements, the fluorometric wave pattern shows that in one of the chromosomes the short arm has a distinctly brighter fluorescence. The fluorescence patterns of the two chromosomes measured fluorometrically on the same intensity scale are given in figure 26.

Chromosome number 19.

Chromosome no. 19 has a very light overall fluorescence compared to the fluorescence of no. 20. Light, narrow fluorescent bands were observed on each arm proximal to the centromere, giving it a symmetrical pattern. Manolov *et al* (1971) described this chromosome as having positive fluorescence along only ± 30% of its total length. The median and distal regions on both arms show dull fluorescence.

In Giemsa-stained preparations, Evans *et al* (1971) observed a dark stained region near the centromere and weak stained areas on the long arms.

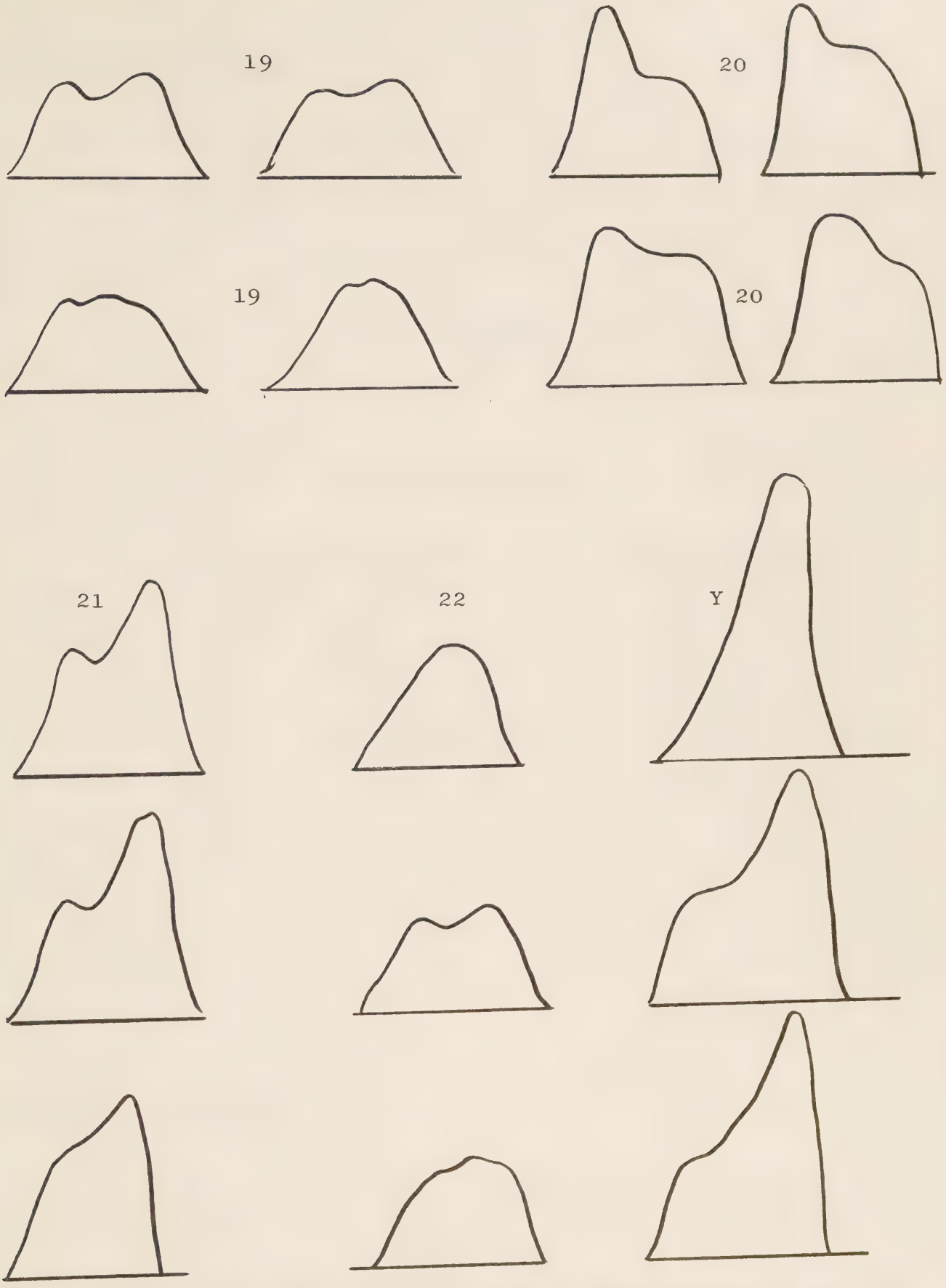

Figure 26 - Wave pattern recordings of chromosomes 19, 20, 21 22 and Y measured on the same intensity scale.

Chromosome number 20.

Chromosome number 20 has an overall fluorescence brighter than that of chromosome no. 19. The short arm has a bright broad band in the median region while the long arm has a light to dull fluorescent area.

In Giemsa-stained preparations the centromere region is not as positive as that of chromosome no. 19. (Evans et al 1971).

GROUP 21 - 22(G)

These acrocentric chromosomes which form the 21-22(G) group cannot be distinguished morphologically but have quite distinct fluorescence. Both chromosomes may have satellites, which are polymorphic in their staining with quinacrine mustard. The satellites can vary in fluorescence from dull to bright and intense; the reason for this is unknown. In addition the long arm of these chromosomes produce constant patterns, and the 4 acrocentric chromosomes can be divided into two distinct classes.

The wave pattern measured photo-electrically on the same intensity scale for chromosomes 21, 22 and Y chromosome are given in figure 26.

Chromosome number 21.

The overall fluorescence of chromosome no. 21 is considerable higher than that of no. 22 (figure 26). The proximal region of the long arm is brightly fluorescing and the distal region appears dull. In studies on these chromosomes in a less contracted state,

Manolov et al (1971) observed bright fluorescence on the proximal and median regions of the long arm, and dullness on the distal regions.

In their autoradiographic studies, Ganner and Evans (1971) found a positive correlation between the bright fluorescence of the chromosome no. 21 and its late DNA replicating pattern. The ASG techniques of Evans et al (1971) gave a positive stain in the proximal two-thirds of the long arm.

Chromosome number 22.

Chromosome no. 22 gives light fluorescence when stained with quinacrine mustard. The long arm appears to have a light band in the median region. The main diagnostic difference between the two G-group autosomes is the light median region of the long arm of no. 22 compared with the bright median region of no. 21.

Giemsa preparation of this chromosome gives a weak stain, with a band visible in the median region of the long arm. (Evans et al 1971).

The Y chromosome.

In those Y chromosomes that are longer than G-group chromosomes the distal region stains intensely with quinacrine derivatives. This intense region is polymorphic and varies in size between different individuals. The short arms and proximal region give a dull fluorescence. A Y chromosome that is longer than a G-group chromosome could be identified immediately on metaphase-figures by this intense fluorescence, while a Y chromosome shorter than a G-

group chromosome was found to be non-fluorescing. (Retief and Van Niekerk 1971).

Manolov et al (1971) studied less contracted Y chromosomes and found that the intense region consisted of two very close bands, q3 and q5. A very narrow, light band was also seen in the proximal region.

In Giemsa-stained banding preparations, two clear Giemsa-positive bands were seen by Evans et al (1971).

The characteristics of Y chromosome fluorescence in metaphase have been studied and the results follow in the next chapter.

CONCLUSION.

It was found that normal human chromosomes stained with quinacrine mustard could all be identified visually in photographic prints that showed strong contrast. The characteristic and reproducible banding patterns of each of the 24 human chromosome types was described.

The value of a photo-electric method to distinguish the relative fluorescent intensity of a chromosome is illustrated by the differences in intensity that were observed in this study, compared to those reported by Manolov et al (1971) who subjectively measured the intensity of fluorescence and described in chromosome nos. 3 and 15, bands of equal intensity seen on the different arms. On photo-electric recordings it was found that the relative intensity of these bands differed consistently and significantly.

A study of the patterns of quinacrine stained fluorescence in chromosomes of Drosophila by Barr and Ellison (1971) showed it to be a promising tool in the analysis of chromosome differences in

evolutionary cytogenetics.

Familiarity with the appearance of normal banding patterns is also valuable in the recognition of abnormalities in the karyotype because the quinacrine fluorescence patterns are not altered by structural rearrangements. Caspersson et al (1970e) described the abnormal B-group chromosome in the 'Cri du Chat' syndrome as a breakage and consequent loss in the region of a bright fluorescent band on the short arm of the no. 5 chromosome. The Philadelpia (Ph) chromosome was designated as no. 22. (Caspersson et al 1970g). The nature of structural X-chromosome aberrations in Turner's syndrome was studied and the possible breakpoints described.

In the cytogenetic laboratory at Tygerberg Hospital (unpublished), deletions and isochromosomes of the X chromosome, D/D translocations, D/G translocations and trisomics G, D and E is under study using quinacrine fluorescence together with the knowledge of the normal banding patterns described. Whatever the rearrangement, it appears that the banding patterns described in previous paragraphs do not alter, so that a knowledge of the normal karyotype would permit the identification of any structural rearrangement in the karyotype. The nature of some anomalies, however, especially those involving small regions without a distinct fluorescent pattern, will be difficult to explain.

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C H A P T E R III

AUTOMATION IN THE IDENTIFICATION OF HUMAN CHROMOSOMES.

Systems have been devised in the past to use instruments and computers to analyze the human chromosome complement. (Neurath et al 1970, Frey, 1969, Mendelsohn et al, 1969 and others). In clinical Cytogenetics a vast amount of material has to be examined, yielding a relatively small number of interesting cases. Ever increasing interest in research on this subject, together with a growing volume of clinical work, have intensified the need for automatic karyotyping and have given added impetus for developing of systems for this purpose.

The limitations of visual chromosome analysis have already been referred to. It is a time consuming task, fatiguing, subjective and non-quantitative. Systematic measurement by machines is able to reveal far more information than can be discovered by the qualitative judgements of the human operator. In the recognition of form, the human eye and brain are superior to any machine that has ever been developed: machines on the other hand, have a superior measuring capacity.

With the discovery that human chromosomes stain with specific banding patterns after various pretreatments, an enormous amount of detail has suddenly become available to describe each chromosome in the normal karyotype. The qualitative demands of karyotyping have therefore increased.

In an attempt to provide an alternative to visual karyotyping and to improve chromosome identification methods, a chromosome identification computer program - CROTES - was devised in this study based on pattern measurements of normal chromosomes stained with quinacrine mustard.

Quantitative measurements of chromosome-patterns.

The wave patterns recordings (figure 7 - 25) were considered to form the basis for an automated chromosome recognition program. The intensity measurements of the patterns describe the chromosomes quantitatively.

Intensity measurements: The length of the chromosomes varied considerable from cell to cell due to different degrees of chromosome contraction in different cells; and a correction was neccessary to allow for such variation. To normalize the length of the chromosomes, each graph or pattern was divided in 20 segments of equal length on the X-ordinate, and the corresponding Y-values, (intensity values), were read in arbitrary units.

To allow for scale differences between chromosomes, and also the scale setting on the X-Y recorder, the Y-values of a chromosome were transformed by dividing each value by the average of all the readings on that particular graph. The resultant 20 intensity values were used to describe the fluorometric pattern of a given chromosome.

The measurements of intensity in a chromosome were therefore standardized, making it possible to compare differences of intensity between bands on the same chromosome. The great differences

in intensity that exist between different banding regions on chromosomes in the same cell, make the comparison of intensities between chromosomes a difficult task. Obtaining a scale setting on the X-Y recorder to give good differentiation between the bands of especially the poorer staining chromosomes, as well as having a pattern for the intense bands, was impossible.

When selecting a range of patterns within each individual chromosome number, an attempt was made to be unbiased. It was argued that the chromosomes that showed the best differentiation between the bands would be those with the highest variance measurements of their Y-values. The variances of the Y-values are expressed as:

$S^2 = {}^1/19 \sum_{i=1}^{20} (X_i - \bar{x})^2$ and was determined to describe a pattern.

A group of 25 patterns with the highest Y-value-variances was selected to represent each chromosome in the karyotype. In the groups 8, X and 10, however, only 15 patterns could be confidently selected as representative of these groups. Difficulties were encountered in the selection of these chromosomes in metaphase because of their nearly similar length measurements and wave patterns.

A second criterion was used to examine the selected chromosomes for normality:- the 20 intensity values of each chromosome pattern in the group were measured against all the other chromosomes patterns in that group by the following formula:

$$\text{ERROR} = \sum_{i=1}^{n} \sum_{j=1}^{20} (\text{CHRT}_j - \text{CHR}_{ij})^2 \quad \text{where}$$

ERROR = selection criterion

CHRT = chromosome pattern under selection

CHR_i = i-th chromosome pattern against which a given chromosome pattern was tested.

n = number of chromosome patterns in the group.

The ERROR values were calculated for all the patterns within a group. The pattern in a group with the smallest ERROR value represents an average pattern within that group. Deviations from this pattern were then of three types:-

(a) A group that had a better differentiated pattern.
(b) A group that had poorer differentiation of the bands.
(c) A group that differed from the average because of an aberrant pattern not belonging to the group, or because of a technique artifact.

Chromosome patterns that fitted into categories (b) and (c) were selected against and disregarded. The calculation of the ERROR value of each pattern was then repeated against the rest of the patterns. After examination of the patterns in groups (b) and (c) the process was repeated if necessary.

All the normal chromosome patterns were tested and 15 were finally selected to represent the typical patterns of the normal chromosome no. 1 to 18 and the X chromosomes.

In the 13-15(D) group chromosomes the short arm and satellite regions vary in length between different individuals and only the long arm intensity measurements were made.

The fluorescence patterns of chromosomes nos. 19, 20, 21 22 and Y were considered to be too small for intensity measurements. Visual identification of the smaller chromosomes in group F and G can easily be made by observing their staining with quinacrine

mustard, and depends only on an ability to distinguish between bright positive or light positive staining. Studying fluorescent intensity in these chromosomes yielded little information, and patterns for them were not measured.

The means and variances of the 20 intensity measurements of the 15 normal chromosomes were calculated for chromosome numbers 1 - 18 and the X chromosome. The results are given in Tables 1 to 7. The intensity measurements of each of the 15 normal selected patterns for chromosomes nos. 1-18 and X chromosome are given in Appendix 1-19.

Variations in intensity measurements:

High variations in intensity measurements were generally observed in the transition regions between bright and dull fluorescent bands. These regions mainly include the centromere regions and the measurements proximal to intense fluorescent bands such as those found in the no. 3 chromosomes.

Secondary constrictions have an effect on the total length of the chromosomes because of unequal coiling. Intensity measurements which were recorded at equal distances along the length of the chromosome, therefore show high variations in these chromosomes - mainly nos. 1, 9 and 16.

The average fluorescent wave patterns of the normal chromosomes.

The average wave patterns compiled from the mean standardized intensity measurements, recorded photo-electrically, are given in figure 27 for the chromosome nos. 1-18 and the X chromosome.

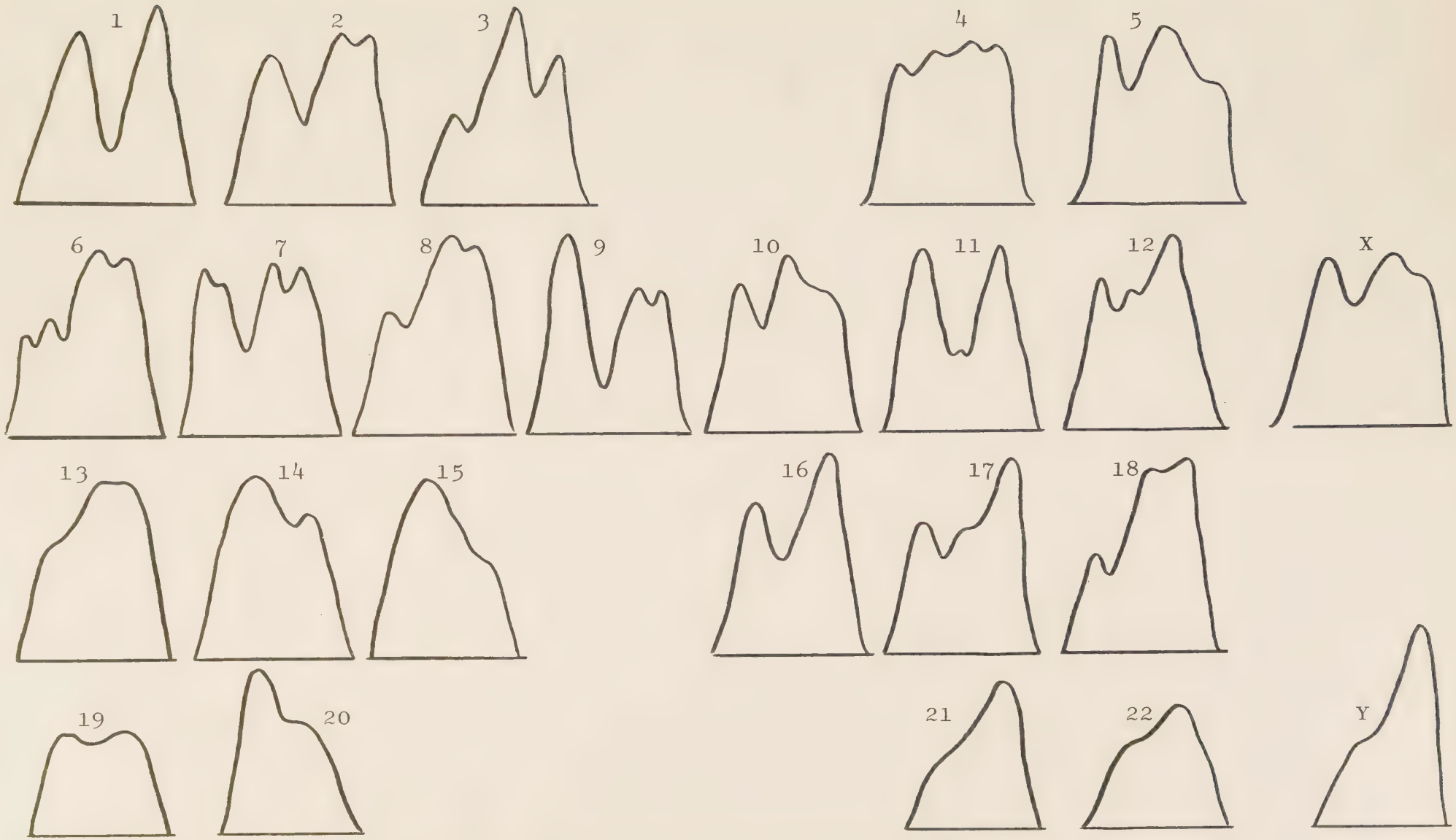

Figure 27 - Average fluorescent wave patterns compiled from the mean intensity measurements for the normal human karyotype.

These average patterns are drawn on the same length scale for all chromosomes. With the exception of chromosomes 3 and 6, the average wave patterns of the normal chromosomes 1-18 and X chromosome correspond with the findings of Caspersson et al (1971a).

The positive regions of the short arm of the no. 3 chromosome were generally found to stain with considerably less relative intensity than the long arm. The findings of Caspersson et al (1971a), as well as the idiogram drawn by Manolov et al (1971), gave the short arm positive region the same relative intensity as the median positive region on the long arm. The short arm of the no. 6 chromosome was generally found to consist of two light positive bands, the distal region being of less intensity.

The average no. 15 chromosome patterns show a dull fluorescent distal region compared to the rest of the long arm (figure 27). Manolov et al (1971) describes the positive bands on the no. 15 chromosome as being of the same intensity. These measurements of Manolov et al were, however, made subjectively.

Comparison of the average wave patterns within the karyotype. (Program AVTEST).

In order to determine which chromosomes in the karyotype have similar patterns which may lead to misidentification in an automated recognition program a test was devised to compare the average patterns.

The mean intensity measurements calculated from normal chromosome patterns (Table 1-7) was used and tested against the other patterns by the formula:

Test value (F-value) = $^{1}/_{19} \sum_{i=1}^{20} (CHRT_i - CHRX_i)^2/VART_i$ where:

$CHRT_i$ = the ith value of the intensity measurement of the chromosome pattern (T) with which the test chromosome is compared.

$CHRX_i$ = the ith value of the intensity measurement of the chromosome pattern under test.

$VART_i$ = the ith value of the variance of chromosome pattern T.

To determine the statistical significance in the comparison of two patterns, Fishers F-distribution can be applied to the formula with 19 and 280 degrees of freedom.

The F-values for 19 and 280 degrees of freedom are:

$F_{,05} = 1,63$ and $F_{,01} = 1,97$.

The variance values of each intensity measurement was used to ~~used to~~ weigh the points. Some intensity measurements were therefore more important in the test because of low variance measurements. A computer program AVTEST was written in FORTRAN language to compare the average fluorescence patterns, Number 19 was designated to the X chromosome in the program. The computer program is given in Appendix 39.

Results:

Tables 8-11 give the test values (F-values) in comparison of the mean intensity measurements of the chromosomes in groups A-E tested against the mean values of the other measured chromosomes in the karyotype.

Group 1 - 3(A)

The comparison of the mean values of chromosome nos. 2-18

and X against the mean values of no. 1, gave significant ($F > 1,63$) differences for all the patterns except nos. 2, 11, 16 and 17. (Table 8).

No significant differences were calculated in the comparison of patterns 7, 16 and 17 with the mean values of chromosomes no. 2.

Chromosome no. 3 differed significantly from all the other patterns in the karyotype except no. 5.

Within the A-group, all the comparisons except one of the average patterns of chromosomes 1-3 differed significantly. In a comparison of the mean values of the no. 2 chromosome against the intensity measurements and variances of the no. 1 chromosome, no significant difference could be calculated ($F=1,09$). The reciprocal test, however, showed a significant difference with a F-value of 2,06.

Group 4 -5(B)

All the average fluorescent patterns in the karyotype differs significantly when measured against the mean values of chromosome no. 4. (Table 8). When testing the values of the X chromosome and number 4 chromosome against those of no. 5 no significant differences could be measured.

Group 6 - 12 and X(C)

The F-values in the comparison of the mean values of nos. 1-18 and X chromosomes against those of the C-group chromosomes are given in Tables 9 and 10. An overlapping occured between the following patterns. (F-values in brackets).

(i) With no. 6 under test: nos. 4(1,36), 8(0,5), 13(1,28) and 18(1,05).

(ii) With no. 7 under test: nos. 2(0,64), 4(1,57), 10(1,56), X(0,72).

(iii) With no. 8 under test: nos. 6(0,84), 13(0,72) and 18(0,56).

(iv) With no. 10 under test: nos. 5(0,90) and X(1,30).

(v) With no. 11 under test: nos. 1(1,27) and 16(1,40).

(vi) With no. X under test: nos. 4(1,28), 5(1,48), 7(1,35), 10(0,66) and 12(1,40).

The chromosome patterns tested against the mean values of chromosomes 9 and 12 differed significantly.

Comparing the mean values of the patterns within the C-group alone, significant differences were calculated between chromosome nos. 9, 11 and 12 in all combinations with the other patterns in the C-group. The chromosome pairs that showed no significant difference between their mean intensity measurements were: numbers 6 and 8, numbers 7 and X, and numbers X and 10.

Group 13 - 15(D)

No statistical significant differences were calculated in the comparison of the mean values of chromosome no. 8 and 18 with the mean values of no. 13. The F-values calculated are 0,93 and 1,45 respectively.

The only chromosome pattern that did not differ significantly from the mean chromosome 14 was no. 15 with a F-value of 0,91. The same was found when the patterns were compared against the mean no. 15; only no. 14 chromosome pattern did not differ significantly.

In the comparison between the mean intensity values of the D-group chromosomes, the average pattern of no. 13 differs significantly from the average patterns of no. 14 and no. 15. The comparison of the means of nos. 14 and 15 showed no statistically difference.

Group 16 - 18(E)

No statistical significant differences were calculated in the comparison between chromosome nos. 1, 2, 11, 12 and 17, and the average pattern of no. 16. In the test against the no. 17 chromosome nos. 2, 4, 6 and 8 showed no significant differences. The no. 18 chromosome pattern was found to overlap with those of nos. 6, 8 and 13.

Comparisons between the mean intensity values within the E-group gave statistical significant differences between chromosome nos. 17 and 18. Between chromosome nos. 16 and 17, however, no significant differences were found when comparing their average banding patterns.

DISCUSSION.

A statistical test was devised to compare the mean intensity measurements of the average fluorescent patterns of the normal chromosomes 1-18 and X chromosome in all possible combinations. Fishers' F-test was applied to determine the degree of similarity between the 19 patterns that may lead to misidentification in an automated computer chromosome recognition program based only on pattern measurements.

The 19 average chromosome patterns were compared in all combinations giving a total of 342 tests. Of the 342 comparisons, which include the reciprocal test between two patterns, 294 (86%) were found to be statistically significant. A total of 171 comparisons were performed if the two tests on a pair of chromosome patterns are considered as one. Of the 171 combinations, 9% were found to have nearly similar patterns judging from the non significant statistical test. These combinations include the following pairs of chromosomes: 1-16, 1-11, 2-7, 2-16, 2-17, 5-10, 6-8, 6-18, 7-X, 8-13, 8-18, 10-X, 11-16, 13-18, 14-15, 16-17.

The mean intensity measurements of chromosome nos. 3, 4, 9, 12, 15 and 18 differed significantly when tested against the mean measurements of the other patterns in the karyotype.

Based on a comparison of the average banding patterns of chromosome 1-18 and X chromosomes, only 6 of the 19 chromosomes could be expected to be identified with certainty in an automated pattern recognition program.

Taking the length and centromere index differences between these chromosomes into account, and classifying the chromosomes subjectively into the groups A, B, C, D, no. 16 and E, twelve of the 19 chromosomes could be expected to be identified with certainty in an automated program. Based on the results of the comparisons of the average patterns, an overlap in banding patterns is expected between chromosome pairs nos. 6-8, 7-X, X-10 and 14-15.

Computer pattern-recognition program, CROTES.

A pattern recognition computer program Crotes, based on the mean intensity values of the normal chromosomes 1-18 and X chromosomes were developed using an IBM 360 computer. The program in Fortran computer language is given in Appendix 40.

The knowledge of chromosome lengths and centromere indexes are important in the use of the program. The visual recognition and classification of human chromosomes in length groups A, B, C, D, no. 16 and E is easy and is being done at first glance routinely in Cytogenetic laboratories since the Denver Conference in 1960. The chromosome being studied is classified in one of the length groups: A, B, C, D, no. 16 and E, before being subjected to intensity measurements.

The mean intensity measurements and variances of the 20 values of chromosome numbers 1 to 18 and the X chromosome (Table 1-7), were punched on cards and used as 'normal' values against which the pattern being studied is tested. The unknown pattern, recorded photo-electrically, is divided into 20 equal distances on the X-ordinate and the 20 corresponding Y-values are determined in arbitrary units. These 20 values are normalized by dividing each value by the average of the 20 values, to account for differences in scale and overall intensity between chromosomes of different cells. The 20 corrected values of the unknown pattern are punched on cards and used in the following formula to determine which chromosome number is the most likely to attach to the unknown chromosome pattern.

$$F = {}^{1}/_{19} \sum_{i-1}^{20} (CHRT_i - CHRX_i)^2/VART_i \quad \text{where}$$

F = Measure of fit of the unknown chromosome pattern X, against the chromosome number (T) tested.

$CHRT_i$ = ith mean Y-value of chromosome pattern, T, against which the unknown pattern X is tested.

$CHRX_i$ = ith Y-value of unknown pattern X.

$VART_i$ = the ith variance of the mean Y-value, i, of chromosome pattern T.

The Y-values of the unknown pattern are tested with the same formula against all the values of chromosome 1-18 and the X-chromosome consecutively.

The resulting 19 F-values and their corresponding chromosome numbers are then arranged in an order of rank, ranging from the lowest to the highest values. The chromosome T, against which the smallest F-value is calculated, is considered to be the most likely one to attach to the unknown pattern. The following number in order of rank is the next most probable chromosome number to attach to the unknown pattern. The number 19 was allocated to the X chromosome.

To determine the statistical significance of the comparison, Fishers' F-distribution can be applied to the folmula with 19 and 20 (n-1) degrees of freedom where n is the number of chromosomes measured to determine the mean intensity values.

The program CROTES is written in Fortran language and given in Appendix 40.

To eliminate the tedious task of dividing the X-ordinate on the graph into 20 equal distances, the X-Y recorder can be linked on line with a computer and the recordings made automatically.

The X and Y terminals of the X-Y recorder used in this study were connected to a computer (Hewlett-Packard No. 2100A) and it was programmed to divide the wave pattern into 20 equal distances and the Y-values were read off accordingly. The procedure for identifying the chromosomes in a metaphase is then simple and involves the following:-

(1) The chromosomes on the metaphase print are numbered arbitrarily. The computer is fed with all the mean and variance-values of the normal chromosomes (Table 1-7).

(2) A chromosome is selected and run on the microscope stage (figure 3), with the light passing through a chromatid from short arm to the long arm telomere.

(3) The beginning and the end values of the chromosome-wave pattern is given to the computer.

(4) The computer automatically divides the X-ordiante in 20 equal distances and reads off the 20 corresponding Y-values.

(5) The 20 Y-values are tested by the computer against all the 'normal' values of chromosomes 1 to 18 and the X chromosomes, and the F-values are calculated.

(6) The computer prints out the 19 F-values and the corresponding chromosome numbers, in order of importance, from the lowest value i.e. the most probable chromosome number to attach to the chromosome tested, to the highest value.

(7) The chromosome number given the lowest F-value is correlated with the length of the chromosome tested, and marked in on the metaphase print.

(8) The statistical significance of fit is determined.

CONCLUSION.

A computer chromosome recognition program CROTES was devised in a system for recognizing and identifying the normal chromosomes

in the karyotype, using only fluorescence banding patterns. Knowledge of the lengths of normal chromosomes is essential in the application of this program.

Testing an unknown chromosome for recognition involves the visual classification of the chromosome into one of the length categories A, B, C, D, no. 16 and E, and the measuring of the relative intensities of the banding patterns after staining with quinacrine mustard. The program CROTES compares the measurements against the patterns of the normal chromosomes from nos. 1-18 and the X chromosome and the possible numbers to attach to the chromosome tested, are given in order of rank.

In the visual correlation of the chromosome numbers given by the computer and the length of the chormosome tested, the correct chromosome number is attached to the chromosome pattern.

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TABLE 1 : Means and variances of fluorescence intensity measurements.

Chromosome 1.		Chromosome 2.		Chromosome 3.	
Mean	Variance	Mean	Variance	Mean	Variance
57,93	327,92	52,40	69,11	47,87	152,27
86,33	495,67	88,47	178,41	94,73	292,92
97,93	174,21	103,80	235,89	121,07	106,92
115,40	226,40	113,07	121,35	126,53	181,70
126,33	196,81	113,00	117,29	114,60	189,26
122,40	325,54	108,60	157,69	100,27	146,07
111,73	300,07	99,80	175,46	96,40	95,97
92,00	227,86	70,47	211,84	107,20	234,74
70,00	384,00	64,73	263,92	126,33	202,38
50,87	312,41	86,87	166,84	161,67	294,67
57,40	386,97	97,73	178,78	153,87	411,41
86,73	366,07	108,07	93,35	155,60	220,97
123,93	288,07	121,53	93,70	108,53	230,41
141,93	200,07	128,73	74,07	90,13	106,98
147,40	156,11	124,87	131,70	78,87	105,70
145,13	286,41	125,47	291,12	74,87	132,70
121,87	212,27	127,93	95,07	86,00	194,57
104,93	355,07	115,07	72,78	87,87	78,70
85,40	249,26	98,53	111,12	68,93	55,35
52,92	204,21	49,67	133,24	37,53	93,12

TABLE 2 : Means and variances of fluorescence intensity measurements.

Chromosome 4		Chromosome 5.		Chromosome 6.	
Mean	Variance	Mean	Variance	Mean	Variance
37,33	33,10	40,20	66,89	43,60	51,97
77,67	108,67	88,13	191,84	75,87	67,84
98,87	128,41	122,67	256,09	83,60	173,40
103,00	56,71	123,73	151,64	79,73	192,64
98,13	102,98	107,80	434,46	81,20	170,03
96,00	152,86	86,07	423,07	91,87	174,84
104,13	245,12	83,60	190,40	84,27	265,35
110,53	70,98	104,27	268,92	76,87	246,84
112,20	57,60	116,80	100,74	98,53	204,84
113,40	27,11	124,00	81,29	119,07	245,64
113,73	37,50	128,93	161,92	123,07	152,92
113,67	35,38	128,00	117,29	130,13	81,12
116,00	69,71	124,13	189,41	130,53	123,55
117,27	73,64	117,00	95,86	126,67	86,10
116,73	70,35	105,93	90,64	127,87	89,55
113,07	64,21	95,47	63,27	128,87	129,12
116,67	49,38	95,33	72,81	125,33	92,10
112,27	41,35	93,33	100,95	117,20	248,74
86,60	91,83	73,87	247,98	96,80	429,03
39,93	77,21	38,80	100,89	57,53	780,98

TABLE 3 : Means and variances of fluorescence intensity measurements.

Chromosome 7.		Chromosome 8.		Chromosome 9.	
Mean	Variance	Mean	Variance	Mean	Variance
50,00	96,57	35,13	69,98	56,73	199,07
106,33	269,95	64,27	77,92	115,80	558,60
124,47	292,84	77,67	36,24	146,40	202,69
117,93	250,78	84,33	70,81	153,60	157,83
113,00	157,29	89,73	83,92	146,40	214,83
108,00	151,71	89,33	101,38	131,07	431,78
95,33	322,24	82,87	55,55	100,80	703,17
72,93	178,64	87,67	192,67	76,33	346,95
70,20	286,74	103,93	327,21	56,73	110,21
90,33	277,81	121,07	159,35	55,53	245,85
109,73	174,92	132,27	85,92	68,80	355,31
129,60	196,11	135,00	75,29	98,00	287,57
118,67	241,24	138,67	104,38	117,00	183,00
110,53	179,12	134,60	53,97	116,00	79,71
118,27	138,50	133,60	80,26	114,13	137,41
123,60	185,97	134,00	105,71	115,07	147,21
110,13	207,55	123,40	63,11	112,80	163,17
100,40	163,69	105,73	90,64	102,53	184,70
85,33	347,38	80,13	161,70	77,13	148,41
45,33	91,10	43,87	71,12	38,20	113,31

TABLE 4 : Means and variances of fluorescence intensity measurements.

Chromosome 10.		Chromosome 11.		Chromosome 12.	
Mean	Variance	Mean	Variance	Mean	Variance
48,20	71,46	35,47	67,84	39,13	94,84
85,47	49,84	76,00	188,71	74,47	100,55
107,20	97,74	109,67	207,95	103,53	54,98
114,60	184,26	134,13	201,84	115,13	106,27
114,00	105,57	138,93	146,92	108,67	139,24
102,00	95,00	132,00	96,57	94,27	85,21
85,13	147,70	112,00	158,86	97,00	174,29
98,40	115,54	79,87	249,55	105,00	66,00
118,60	98,11	72,13	311,55	99,73	108,92
133,40	36,69	74,73	99,78	104,87	37,12
129,20	93,17	71,47	134,41	117,07	85,50
119,67	82,10	89,80	344,17	131,13	74,98
116,27	44,35	116,47	200,27	140,40	90,97
115,33	66,38	131,93	120,64	139,73	71,35
111,27	86,21	143,27	101,92	129,53	99,84
109,60	88,11	138,53	199,27	115,07	105,64
102,73	88,21	120,00	136,71	98,00	118,29
89,00	114,71	100,33	196,75	82,80	163,46
64,33	106,52	76,53	108,12	65,07	114,21
32,73	33,21	44,93	90,50	37,60	46,26

TABLE 5 : Means and variances of fluorescence intensity measurements.

Chromosome X.		Chromosome 13.		Chromosome 14.	
Mean	Variance	Mean	Variance	Mean	Variance
44,80	141,17	32,60	41,83	40,20	43,31
83,87	188,55	56,27	90,64	65,00	55,86
106,87	94,12	73,67	83,95	83,87	81,27
118,80	75,46	86,93	90,92	99,20	81,89
116,33	54,24	93,07	56,21	112,80	91,46
107,33	64,52	97,13	109,12	125,07	51,50
99,73	52,35	99,33	101,10	131,73	23,92
95,13	79,12	105,93	118,64	133,67	26,81
100,07	265,49	117,53	47,84	135,13	92,27
110,13	226,12	125,20	42,89	125,73	58,78
116,07	95,50	132,73	56,35	120,40	98,11
121,73	43,35	134,07	59,35	111,67	55,67
122,00	69,43	135,07	34,64	103,47	35,84
118,93	57,50	132,87	23,98	103,40	299,69
114,87	48,84	133,47	78,84	113,27	126,64
112,00	68,00	125,60	139,11	111,20	136,74
104,87	85,84	115,60	64,69	100,87	86,98
92,07	128,92	95,40	89,54	81,47	64,41
72,80	157,46	68,60	97,97	60,53	55,98
38,80	65,03	37,80	21,17	36,60	44,97

TABLE 6 : Means and variances of fluorescence intensity measurements.

Chromosome 15.		Chromosome 16.	
Mean	Variance	Mean	Variance
46,00	100,00	40,93	73,92
72,27	199,50	69,80	104,31
90,87	157,70	96,00	105,86
107,20	164,74	108,93	152,78
118,80	164,31	115,27	389,35
126,07	175,50	107,07	303,64
135,33	165,81	94,33	341,91
135,07	114,64	82,87	292,98
135,47	169,98	78,47	130,84
133,87	96,98	84,87	99,41
129,53	152,41	91,47	257,84
119,13	93,98	109,13	258,41
108,27	124,64	125,27	213,50
100,40	138,83	146,00	120,14
96,87	113,41	152,27	242,07
91,33	89,10	147,40	312,69
83,13	79,41	130,27	210,35
74,00	70,86	107,13	322,69
58,00	143,86	73,20	119,03
37,13	75,98	41,20	81,46

TABLE 7 : Means and variances of fluorescence intensity measurements.

Chromosome 17.		Chromosome 18.	
Mean	Variance	Mean	Variance
42,47	48,98	34,33	43,95
67,93	105,35	61,47	57,12
95,47	167,84	75,93	65,07
105,73	312,21	83,33	173,24
102,60	423,97	74,13	151,27
92,27	326,21	72,07	235,64
81,13	133,55	78,60	213,83
82,20	226,03	93,40	369,26
92,00	235,86	119,67	139,38
100,27	166,92	133,93	69,07
102,93	176,78	137,40	116,83
106,33	267,24	136,13	123,70
119,67	440,38	134,33	146,38
138,20	312,74	135,53	123,55
148,20	188,60	138,47	123,98
145,67	167,24	141,20	210,89
137,13	208,84	127,80	245,03
112,13	229,84	106,67	186,24
79,33	145,81	71,93	153,35
46,73	97,92	39,80	76,03

TABLE 8: F-values in the comparison of the mean intensity values of the chromosomes numbers 1, 2, 3, 4 and 5 against the mean values of all the measured chromosomes in the karyotype. (NS = non significant, F < 1.63).

CHROMOSOME COMPARED	CHROMOSOME UNDER TEST.				
No.	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.
1.	---	2,06	11,73	18,87	12.07
2.	1,09 NS	---	7,61	6,25	5,65
3.	8,04	8,43	---	13,01	2,71
4.	2,70	1,92	3,83	---	1,56 NS
5.	4,34	3,93	1,60 NS	3,14	---
6.	3,30	2,38	7,79	3,44	4,00
7.	2,09	1,24 NS	4,83	5,76	3,55
8.	3,39	3,34	7,48	3,79	3,97
9.	2,24	3,34	7,69	19,75	8,68
10.	3,80	3,64	1,89	3,08	0,54 NS
11.	0,53 NS	2,07	9,17	12,21	8,20
12.	2,45	3,46	4,73	3,58	1,73
X.	2,38	1,99	2,81	1,79	0,93 NS
13.	3,67	4,39	6,22	3,58	3,03
14.	4,35	6,17	3,91	4,18	2,57
15.	5,86	8,10	2,69	7,52	2,43
16.	0,74 NS	1,28 NS	9,96	7,27	7,89
17.	1,47 NS	1,29 NS	8,96	4,25	6,48
18.	4,72	5,26	8,39	5,78	4,98

TABLE 9.

F-values in the comparison of the mean intensity values of the chromosomes numbers 6, 7, 8, 9 and 10 against the mean values of all the measured chromosomes in the karyotype. (NS = non-significant, F < 1,63).

CHROMOSOME COMPARED.	CHROMOSOME UNDER TEST.				
No.	No. 6.	No. 7.	No. 8.	No. 9	No.10.
1.	6,20	2,99	11,47	2,85	18,5
2.	2,15	0,64 NS	5,52	2,34	7,77
3.	7,81	4,28	14,43	8,60	4,04
4.	1,36 NS	1,57 NS	3,35	5,09	1,98
5.	3,15	1,77	7,33	5,04	0,90 NS
6.	---	2,01	0,84 NS	7,21	4,99
7.	3,01	---	8,19	1,79	5,83
8.	0,50 NS	2,50	---	7,76	4,52
9.	9,34	2,07	22,27	---	16,81
10.	2,37	1,56 NS	5,11	5,30	---
11.	5,72	2,29	11,68	1,69	11,84
12.	2,01	1,64	3,67	4,64	3.24
X.	2,02	0,72NS	5,03	3,27	1,30 NS
13.	1,28 NS	3,01	0,72 NS	8,36	3,74
14.	4,31	3,67	7,85	7,94	3,17
15.	6,24	4,36	11,30	8,48	3,32
16.	2,60	1,86	4,36	4,01	9,15
17.	1,63	1,89	5,81	4,89	6,49
18.	1,05 NS	3,89	0,56 NS	10,09	5,47

TABLE 10:

F-values in the comparison of the mean intensity values of the chromosomes numbers 11, 12, X, 13 and 14 against the mean values of all the measured chromosomes in the karyotype. (NS = non-significant, F < 1,63).

CHROMOSOME COMPARED.	CHROMOSOME UNDER TEST.				
No.	No.11.	No.12.	No. X.	No.13.	No.14.
1.	1,27 NS	10,95	7,92	21,63	18,98
2.	1,96	4,73	2,43	11,11	19,39
3.	13,11	11,32	5,59	15,24	10,23
4.	4,40	1,98	1,28 NS	3,52	6,41
5.	6,78	2,42	1,48 NS	5,24	11,69
6.	6,36	4,18	4,82	3,59	19,16
7.	2,84	4,23	1,35 NS	11,43	18,68
8.	6,39	2,96	4,62	0,93 NS	15,82
9.	2,27	13,37	6,72	28,54	26,77
10.	6,07	2,73	0,66 NS	4,65	9,41
11.	---	7,38	6,14	16,78	16,36
12.	3,94	---	1,40 NS	3,03	9,53
X.	3,32	1,70	---	4,62	8,40
13.	6,38	2,64	3,60	---	8,14
14.	6,07	5,51	4,09	7,08	---
15.	8,41	6,80	5,18	9,60	0,91 NS
16.	1,40 NS	3,85	4,73	8,75	15,86
17.	3,01	3,32	4,74	5,81	17,50
18.	9,34	4,93	7,48	1,45	17,76

TABLE 11:

F-values in the comparison of the mean intensity values of the chromosomes numbers 15, 16, 17 and 18 against the mean values of all the measured chromosomes in the karyotype. (NS = non-significant, F < 1,63).

CHROMOSOME COMPARED.	CHROMOSOME UNDER TEST.			
No.	No.15.	No.16.	No.17.	No.18.
1.	14,49	1,59 NS	2,74	14,08
2.	9,98	1,11 NS	1,24 NS	7,33
3.	2,80	9,90	7,36	9,46
4.	4,27	2,29	1,51 NS	2,93
5.	3,21	4,21	3,15	4,94
6.	9,31	2,50	1,05 NS	1,54 NS
7.	7,90	2,46	2,25	8,85
8.	8,05	2,32	1,06 NS	0,46 NS
9.	13,13	5,09	5,32	21,14
10.	2,71	3,88	2,55	3,87
11.	11,19	0,98 NS	1,86	11,01
12.	4,39	1,59 NS	1,63	3,27
X.	3,48	1,97	1,69	4,43
13.	5,03	3,09	1,91	0,66 NS
14.	0,81 NS	5,16	4,27	4,48
15.	---	6,85	6,28	6,23
16.	11,45	---	0,37 NS	5,58
17.	11,02	0,43 NS	---	3,25
18.	9,24	3,72	1,68	---

CHAPTER IV.

ACCURACY OF IDENTIFICATION OF CHROMOSOMES BY COMPUTER PROGRAM CROTES.

Using conventionally stained preparations of human chromosomes, Mendelsohn et al (1969), Ruddle et al (1970) Neurath et al (1970) and others describe representative efforts at automating human chromosome analysis. Neurath et al (1970) described a computer system to both measure and classify human chromosomes, from a 35 mm. photomicrographic negative, automatically into 10 classes with 99% accuracy. These 10 classes were numbers 1, 2, 3, B, C including X, D, 16, E, F and G. Mendelsohn et al (1969) developed a method based on optical density measurements of the DNA content as a parameter for karyotype analysis.

In all these approaches the chromosomes are treated in black and white and the methods restricted to size, shape and total DNA content as parameters for karyotype analysis.

With the discovery of specific banding patterns on human chromosomes after staining with quinacrine mustard a new parameter, the fluorescence intensity measurements on chromosomes, are available to describe a chromosome. A computer system was devised to measure the fluorescent intensities in a photomicrographic print and to classify the chromosomes mathematically into 19 groups. These groups are chromosomes numbers 1 to 18 and the X chromosomes. The remaining 5 smaller chromosomes are easy to recognize indivi-

Table 12: Classification of 285 chromosome patterns identified by computor program CROTES.

Number of chromosomes tested.	Chromosome no.	Number classified correctly	Mean F values	Next most probable chromosome no.	Next most probable chromosome with F-values F	
Group A:						
45	1	15	0,90	2	4,27	
	2	13	0,98	1	1,64	
	3	15	0,98	1	8,65	
Group B:						
30	4	15	0,98	5	2,15	
	5	15	1,08	4	5,41	
Group C:						
120	6	14	1,02	8	3,14	
	7	15	0,97	X	3,83	
	8	8	1,05	6	1,15	NS
	9	15	0,98	7	3,32	
	10	15	1,13	X	1,81	
	11	15	0,95	9	2,55	
	12	15	1,06	10	4,41	
	X	8	0,88	7	1,33	NS
				10	2,72	
Group D:						
45	13	15	0,94	14	9,38	
				15	5,70	
	14	12	1,10	15	1,48	NS
				13	8,58	
	15	15	0,97	14	3,10	
				13	11,40	
Group E:						
45	16	11	1,01	17	1,38	NS
	17	15	0,96	18	5,01	
	18	15	0,99	17	2,43	

dually on their overall staining intensity.

In order to test the validity of the computer program CROTES, 285 chromosome patterns were selected visually from normal metaphase figures, and tested in a program for automatic recognition of the patterns. The chromosome patterns were selected visually by their banding patterns, general morphology and by a process of elimination. The visual identification of these chromosomes was performed as is described in Chapter II.

RESULTS AND DISCUSSION.

Table 12 and Appendix 20-38 gives the classification of the 285 chromosome patterns made by computer program CROTES. In Table 12 the mean of F-values in the statistical test of the 15 chromosomes, is given as well as the mean F-value for the chromosome given by the computer as being the next most probable number, to attach to the patterns.

The accuracy of identification of the patterns by program CROTES is indicated by the number of patterns correctly identified, and the mean F-values of the statistical analysis of the patterns. The mean F-values of the next most probable chromosome, against which the patterns were classified, are also an indication of the distinguishing features of the pattern concerned. The higher the mean F-value the greater the deviation of the chromosome patterns under test from the next most probable pattern.

Group 1 - 3(A)

The 45 fluorescent banding patterns recorded photo-electrical-

ly in group A chromosomes, were tested by pattern recognition program CROTES and classified under the three chromosome numbers 1, 2 and 3.

The 15 no. 1 patterns were all correctly identified with a mean F-value of 0,90. The mean F-value fitting these chromosome patterns against the mean no. 2 pattern values differed highly significantly at the 99% level with a value of 4,27.

All the nos. 2 chromosome patterns except 2 were identified and correctly classified with a mean F-value of 0,98. The mean F-value, when these 15 patterns were fitted against the no. 1 pattern, was 1,64, which is significant. Two patterns were classified with the no. 1 chromosome as first choice. The F-values of these patterns nos. 8 and 9 in Appendix 21, were 1,32 and 0,96 when fitted against the mean values of the normal no. 2 chromosome. Neither pattern differed significantly from the mean ($F_{,05}$ = 1,63).

No other chromosome in the normal karyotype has a pattern nearly similar to the no. 3 chromosome. The 15 patterns of chromosome no. 3 which were identified by program CROTES fitted with a high degree of accuracy. (Mean F= 0,98). The next most probable chromosome, no. 1, for these patterns, differed highly significant with a F-value of 8,65.

Group 4 - 5(B)

To test the validity of computer program CROTES for the identification of chromosomes by their banding patterns, 30 B-group chromosome patterns, believed to be 15 no. 4 and 15 no. 5 chromosomes, were measured and tested for recognition by the computer program.

The 30 chromosome patterns were classified into two groups of 15 each: The F-values, as a measure of accuracy fit, were found in the test of the no. 4 chromosomes to range from 0,4 to 1,72 with a mean of 0,98. One pattern differed significantly from the mean. When these 15 patterns were tested against the mean intensity and variances of the no. 5 chromosome, a highly significant mean value of 2,15 was calculated.

The 15 no. 5 chromosomes were all identified correctly, with only one pattern differing significantly from the mean. (No. 3 - Appendix 24). Measured against the no. 4 values these patterns differed significantly with a mean F-value of 5,41.

Group 6 - 12 and X(C)

In this group 120 patterns were tested by the computer for classification under chromosome nos. 6-12 and X.

In chromosome nos. 7, 9, 10, 11 and 12 all patterns were identified correctly. The patterns in these groups fitted with a high degree of accuracy. In all cases, when it was attempted to fit the patterns against the next most probable number, as selected by the computer, there were significant discrepencies.

Judged from a comparison of the average pattern values (Tables 8-11) the patterns of the following pairs of chromosomes were expected to overlap: chromosomes number 6 and 8, and the average patterns of no. 7 and the X-chromosomes.

Chromosome 6 and 8 identified by CROTES.

Identification of the chromosome no. 6 by CROTES was highly successful, with 14 out of 15 being classified correctly.

F-values range from 0,29 to 1,96, with two patterns differing significantly from the mean. The mean F-value was 1,02.

When the 15 no. 6 patterns where fitted against the next most probable chromosome, no. 8, there was a highly significant difference with a mean F-value of 3,14. When the average patterns were compared, it was found that the no. 6 did not differ from the no. 8 pattern (Table 9). Testing 15 no. 6 chromosomes by CROTES showed that in 99% of cases no. 6 chromosomes was given as first choice.

When classifying the no. 8 chromosomes by CROTES only 8 out of 15 tested were classified correctly. All patterns, however, fitted accurately with a mean F-value of 1,05. When the 15 no.8 patterns were tested against the no. 6 mean values no statistically significant difference could be calculated. (F = 1,15).

When identifying normal chromosomes in the karyotype it appears to be advisable to select the no. 6 chromosomes first, and to consider the two remaining chromosomes, which could fit either pattern, as no.8 chromosomes. If the computer program CROTES then identifies 4 chromosomes in a karyotype as no.6 and does not identify a no. 8, the karyotype should still be considered as being normal because of the overlap in the mean measurements of these two chromosomes.

Chromosome no. 7 and the X chromosome.

When comparing the intensity values of 15 X chromosomes against the mean values and variances of the no. 7 chromosome, no statistically significant differences was calculated. The mean F-value calculated was 1,33. Program CROTES classified 8 out of the 15

X chromosomes correctly. All the X chromosome patterns, except one, fitted with a high degree of accuracy against the mean of the normal X pattern. However, the same was found when they were tested against the number 7 chromosome.

When comparing the 15, no. 7 chromosomes with the average X chromosome pattern, a highly significant deviation was calculated with a mean F-value of 3,83. All of the 15 no. 7 patterns were identified correctly by computer program CROTES.

Usually in the analysis of the karyotype, no. 7 chromosomes that differs significantly from other patterns should first be identified definitely as being the true no. 7 chromosomes. The 'false' no. 7 chromosomes would then be known to be the X-chromosomes and would also fit accurately against the mean of the X chromosome.

Chromosome no. 10 and the X chromosome.

The 15 no. 10 chromosomes tested by computer program CROTES were identified correctly. The same patterns when fitted against the mean of the X chromosome, differed significantly on the average with a mean F-value of 1,81. Although some no. 10 patterns may fit the average X chromosome pattern with a value that is non-significant, the F-values found when fitting them against the mean no. 10 values, are lower and therefore ranked higher.

Group 13 - 15(D)

Chromosome no. 13 has a long-arm fluorescence pattern that differs greatly from all other patterns in the karyotype. The 15 no. 13 chromosomes tested and classified by computer program

CROTES were, therefore, identified perfectly accurately.

Chromosomes 14 and 15.

Of the 15 no. 14 chromosomes subjected to testing by the computer program only 12 were correctly identified. When compared with the mean values of no. 15 they did not on the average differ significantly. (F = 1,48).

The 15 no. 15 chromosomes, on the other hand, when tested against the mean values of chromosome no. 14 differed significantly. All no. 15, with mean F-value 3,10, were correctly identified by computer program CROTES with a high degree of accuracy. (Mean F = 0,97).

When using computer program CROTES to identify the D-group chromosomes, it is therefore preferable to adhere to the following order: viz. numbers 13, 15 and 14.

Group 16 - 18(E)

Only 11 out of the 15 no. 16 chromosomes were classified correctly by CROTES. The patterns of chromosome no. 16 did not differ significantly from those of the chromosome no. 17.

The no. 17 chromosomes on the other hand were all classified correctly by the computer program. When testing them against the no. 18 chromosomes, there were highly significant differences with a mean F-value of 5,01.

No other chromosome in the normal karyotype has a pattern nearly similar to that of the no. 18 chromosome. When tested against the no. 17 pattern a significant average F-value of 2,43 was calculated for the 15 patterns measured.

CONCLUSION.

The degree of exactness achieved in identifying the normal karyotype by computer program CROTES, based only on wave patterns of the fluorescent bands on the chromosomes, can be considered highly successful. Of the 285 patterns of normal chromosomes tested and classified by the computer program, 261 (91%) were classified correctly under one of the 19 chromosome numbers.

In groups A and B all the chromosomes were identified correctly. It was found that the sequence of identifying the chromosomes nos. 6, 7, 8, 10 and X in the C-group is important. In the case of these chromosomes it is advisable to karyotype them in strict numerical order beginning with no. 6 and ending with the X chromosome as the last to be identified. The nos. 9, 11 and 12 have patterns that differ highly significantly from all other patterns in the C-group. Overlapping of nos. 8 and X chromosomes with nos. 6 and 7 respectively, should be considered as normal.

In the D-group chromosomes, after identifying the numbers 13 and 15, some number 14 chromosomes may be classified as number 15. When using computer program CROTES to identify the D-group chromosomes, it is therefore preferable to adhere to the following order: viz. numbers 13, 15 and 14.

In the E-group the no. 16 chromosome pattern and the no. 17 pattern are very similar. The no. 16 can, however, easily be identified on visual recognition of the median placed centromere. The recognition of the morphologically similar nos. 17 and 18, using the fluorescence banding technique and computer program CROTES is 100% accurate.

Translocations, deletions and normal polymorphisms causing a change from the normal intensity measurements within a chromosome or chromosomes, would be recognized by the pattern recognition program CROTES. A chromosome number with a pattern that is nearest in form to that of the abnormal chromosome would, however, be allocated to the pattern under test.

These deviations would cause the computer program to identify certain chromosomes in triplicate while in others only one of the normal pair would be identified. The occurence of these arrangements in more than one metaphase cell tested of an individual, would indicate a probable translocation or polymorphism in the staining with quinacrine mustard.

It is possible to use the information obtained from the normal intensity measurements to construct a range of intensity values for known balanced translocations and deletions. This would be easy in Robertsonian translocations and in iso-chromosomes. In balanced translocations the defining of the breakpoints could, however, lead to erroneous results. The intensity information of a hypothetical translocation could be incorporated in the program CROTES and used to test the occurrence of a suspected translocation. It would be advisable to test the average pattern of a hypothetical translocation against the average patterns in the normal karyotype by program AVTEST. By means of this comparison, the patterns which may lead to misidentification in the recognition program CROTES are revealed.

Metaphase figures with chromosomes in trisomy or monosomy would be recognized by computer program CROTES. The final results in the recognition program would reveal an extra chromosome in trisomics and in the case for monosomics only one chromosome of a pair

would be identified.

Caspersson et al (1971b) used a series of 11 pattern curves from each of the 8 chromosome types to demonstrate the statistical validity of the individual patterns. The statistical analysis based on the Fourier parameters of the 87 patterns showed a significant separation of the patterns. Møller et al (1972) reported that they could identify 88% of the chromosomes correctly in a recognition system based on the Fourier analysis of the patterns.

The application of the automated recognition system based on pattern measurements will be especially valuable in the screening of large numbers of chromosomes for abnormalities. The accuracy of identification by computer program CROTES is high and with the interaction of a skilled technician can be 100%.

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Figure 28a - The Y chromosome seen in interphase cells. (a) F-body of a short Y chromosome present in a lymphocyte. (b) Duplex F-body in a lymphocyte. (c) F-body in a polymorpho-nuclear leucocyte. (d) F-body of a long Y chromosome.

Figure 28b - The Y chromosome seen in the sex vesicle in prophase I of meiosis and two F-bodies present in a spermatocyte of an XYY male.

C H A P T E R V.

THE Y-CHROMOSOME FLUORESCENCE IN INTERPHASE CELLS.

In metaphase cells of the normal male, the distal end of the long arm of the Y chromosome fluoresces intensely when stained with quinacrine mustard (Zech, 1969). It has subsequently been shown by Pearson et al(1970), Caspersson et al (1970) and George (1970) that it is possible to detect the presence of the Y chromosome in interphase cells from various tissues. In the interphase cells of a 46 XY individual; the Y chromosomes appear as a single or sometimes double (duplex) bright spot. (Figure 28). In an extremely short Y chromosome, however, Retief and Van Niekerk (1971a) and Borgaonkar (1971), demonstrated a loss of bright fluorescence on the distal end of the Y chromosome and no F-body fluorescence in interphase.

The application of this technique in routine examination for the presence of the Y chromosomes in interphase cells has been found to be reliable. Polani and Mutton (1971) using quinacrine mustard, and Retief and Van Niekerk (1971b) using quinacrine dihidrochloride, demonstrated successfully the use of these staining techniques in the identification of Y chromosomes in various tissues.

In an attempt to test the suitability of this technique for a routine survey of Y chromosomes in the normal newborn population, a pilot study was undertaken to examine the staining behaviour of the Y chromosome in normal and abnormal chromosome complements of males and females. The F-body was examined in interphase cells

of males with a XY, XYY and XX chromosome complement, as well as in females with a 46 XY complement (testicular feminizing syndrome). With the results of this pilot study as a guide, a survey of the newborn population for the detection of Y chromosome abnormalities was undertaken and is still in progress. The study was initiated in January 1972, and at the time of writing, (September 1972) 2039 newborn babies have been screened for the presence of Y chromosomes through examination of blood smears.

A. Y CHROMOSOME FLUORESCENCE IN THE ADULT POPULATION.

Material.

To examine the staining behaviour of the Y chromosome in various tissues, preparations were obtained from the following:

(a) Normal individuals:

Blood smears and buccal smears were taken from 10 males and 10 females with a normal chromosome complement with the males having a Y chromosome of normal average length.

Blood smears and buccal smears were also taken from a normal male with complaints of infertility and a short Y chromosome.

Fibroblasts from normal males were grown in culture on coverslips for the examination of F-body fluorescence.

Amniotic cells obtained from 20 amnion specimens at 38 gestation were prepared for the presence of an F-body and the results correlated at birth with the sex of the baby.

(b) Cytogenetic abnormal individuals.

Blood smears and buccal smears of a 47 XYY male, a 46 XX male and two females with a 46 XY chromosome complement, were taken for examination of the Y chromosome by fluorescence studies.

Techniques.

The preparations of blood smears and buccal smears were fixed in methanol and fibroblasts and amniotic cells in 3:1 ethanol-glacial acetic acid. The fixed slides were allowed to dry, washed in alcohol steps and stained with 0,5% solution of quinacrine dihidrochloride. After staining, the preparations were mounted in fresh buffer (Mac Ilvaine's at pH 4,5) and sealed with nail varnish.

The slides were examined through a Zeiss fluorescence microscope with exciter filters BG12 and barrier filter combination 53/44. The examination for F-bodies were made on coded slides.

RESULTS.

(a) The F-body in normal individuals.

The Y chromosome could be seen as a brightly fluorescent body (F-body) in interphase lymphocytes and buccal epithelial cells of normal males. (Figure 28). Unbiased counts were performed on coded slides of 20 individuals, and the sex noted. The counts of cells with an F-body, (positive) and without the F-body, (negative) are given in Tables 13 and 14 for lymphocytes and buccal epithelial cells. The sex was correctly determined by the F-body count in all smears taken from normal individuals. Of the 300 lympho-

cytes from males 64% were positive for the presence of an F-body. In cells of female origin a body, similar in appearance, was seen in 2% of the 300 cells examined. Of the 500 epithelial cells, 67% of cells from normal males were positive and 9% of female cells. A very low percentage of double F-bodies (duplex-bodies) were observed in cells from normal males.

Short Y chromosome:

In 50 buccal epithelial cells and 30 lymphocytes from a male with a short Y chromosome, shorter in length than a G-group chromosome, no typical F-body was seen and the slides were classified as being of female origin.

Amniotic cells:

In the 20 cell preparations obtained from amniotic fluid taken at 38 weeks' gestation, 14 were classified as females and 6 as males according to the presence of F-bodies in the amniotic cells. Subsequent correlation with the sex at birth showed 11 were female and 9 male. In three preparations the slides were interpreted incorrectly. These males has a normal-sized F-body in blood smears. The F-body counts of amnion cells are given in Table 15 for the groups.

(b) The F-body in cytogenetically abnormal individuals.

The 47 XYY individual:

In 50 buccal epithelial cells, 33 cells were counted as having two distinct F-bodies. In 8 cells only one F-body was seen and

there was no typical fluorescence in the remaining cells. The 30 lymphocytes examined showed 23 with double F-bodies, 3 with only one F-body and 4 with no F-bodies. These results were independently repeated and nearly similar counts were obtained.

The XX male:

In fluorescence studies for the presence of F-bodies in blood smears and buccal smears of a male individual with a 46 XX chromosome complement, the results were negative and the cells classified as being female. No typical Y chromosome fluorescence was observed in metaphase or interphase cells from this individual.

Testicular feminizing syndrome and 46 XY chromosome complement.

In a study of blood smears and buccal smears from two females with a 46 XY chromosome complement, the cells were unbiasedly classified as being of male origin. The F-body counts were 40% and 47% in blood smears of the two individuals and 51% and 58% in buccal smears. The F-body was of similar appearance to that in males with average length Y chromosome, and subsequent metaphase studies of Y fluorescence showed normal Y chromosomes with distinct distal fluorescence in the two females with 46 XY chromosome complements.

DISCUSSION.

This pilot study has shown that the application of the F-body technique in clinical and chromosome diagnosis, and for prenatal detection of Y chromosomes in amniotic cells, seems to be as promising as the earlier identification of the Barr-body in interphase-

cells. The evidence that the fluorescent body represents the Y chromosome is based on the bright Y chromosome fluorescence at metaphase and the absence of this fluorescent body in females, in males with a 46 XX complement, and in males with a short Y chromosome. The presence of two F-bodies in males with two Y chromosomes further substantiates this finding.

Examination for the fluorescent F-body was done more easily in lymphocytes of blood smears than in epithelial cells of buccal smears, because of bacterial fluorescence in the latter.

When using quinacrine dihidrochloride, a count of 30 blood lymphocytes was found to be adequate to differentiate between individuals who have Y chromosome containing cells and those without the Y. A negative finding in male patients, however, has to be interpreted with caution because of non-fluorescence in small Y chromosomes.

In the study of amniotic cells obtained at 38 weeks gestation, the technique was found to be unreliable in 3 of the 20 cases examined. The subsequent blood lymphocyte examination of these three cases revealed the presence of normal F-bodies. The detection of F-bodies for purposes of sex determination in antenatal counselling is more important at 14-18 weeks of pregnancy. At this stage of pregnancy the family who has a history of sex-linked disorders are anxious to know the sex of the fetus. It is, however, difficult to obtain cells at this gestational age for experimental purposes. Before extensive application of this technique on amniotic cells a study of the reliability with respect to uncultured amniotic cells at various gestational ages should be undertaken. A Barr-body identification should also be mandatory in these situations.

B. Y CHROMOSOME FLUORESCENCE IN THE NEWBORN POPULATION.

The frequency of Y-chromosome abnormalities in newborn populations is still uncertain because of the past lack of a simple and accurate technique for screening large numbers of infants. With the discovery of Y chromosome fluorescence in interphase, large populations can be screened quickly and reliable for the presence of an extra Y chromosome or abnormalities involving the Y chromosome.

A study of F-body fluorescence in lymphocytes of all babies born in Tygerberg Hospital was undertaken in Januarey 1972 and is still in progress. The objects of this study are to determine the suitability of this technique for routine surveys and to determine the frequency of occurrence of abnormalities involving the Y chromosome and the F-body.

Material and methods.

Cord blood is taken sterile at birth of all babies born at Tygerberg Hospital since January 1972. The study is still in progress and at the time of writing 2039 babies have been examined.

Blood smears are made as soon as possible after receipt of the specimen, and slides fixed in methanol for at least 30 minutes. The smears are stained in quinacrine dihidrochloride and examined as described in the preceding paragraphs.

The slides are coded, and examined unbiasedly for the presence of F-bodies, and the results correlated with the sex of the baby.

The following counts of F-bodies in lymphocytes are considered as normal for males and females:

(a) Normal males:

A positive F-body count of 20% or more is classified as normal. Generally 30 cells are counted and the presence of 6 or more cells with F-bodies, regarded as normal. Double F-bodies of equal size present in 3 out of 30 cells, are within the bounds of normality. In low F-body counts and in high proportions of double F-bodies, the slides are classified as abnormal.

(b) Normal females:

A positive count of up to 3/30 F-bodies in smears are being classified as normal for females. Higher counts in female lymphocytes are classified as abnormal.

In all abnormal findings a blood culture for chromosome study is being set up and subsequently examined for the presence of the Y chromosome in metaphase.

Babies with an abnormal chromosome complement are being followed-up and the F-body and chromosome investigation confirmed. Careful clinical examinations are being performed on the babies at this stage. In the migrant population that we are dealing with, the follow-up is both difficult and time consuming.

RESULTS.

Abnormal findings in F-body fluorescence in the newborn population fall into 3 categories:-

(i) Males with a low F-body count (below 20%).

(ii) Males with a high incidence of normal-sized

double F-bodies (above 10%).

(iii) The only abnormality noted in cells of female origin is a high count (above 3%) of F-bodies in the lymphocytes.

Of the 2039 babies examined 1053 were males and 987 females.

Male-population.

Normal: Of the 1053 males, 1007 (95%) were classified as normal on the examination of the F-body in lymphocytes of the first slide.

A count of 13 positive cells (43%) of the 30 counted was found to be average for normal males.

Low F-body count: 38 of the 1053 (4%) males were classified as having a low F-body count (below 20% F-bodies), on examination of the first slide. Fourteen of these (1% of total) were repeated and found to be normal at the second count on a repeat smear. These mis-classifications can be ascribed to technical difficulties.

Of the remaining 24 males with low F-body count, 5 have subsequently been shown to have a Y chromosome with a very small fluorescent region. The F-bodies found in these patients were either very small or were interpreted as other fluorescent chromatin in the examination. In 19 male individuals with low F-body counts, the chromosome studies are still in progress.

Double F-bodies: Of the 1053 males examined for F-body fluorescence 6 were found to have a higher than normal percentage (above 10%) of two equal-sized F-bodies present in the lymphocytes.

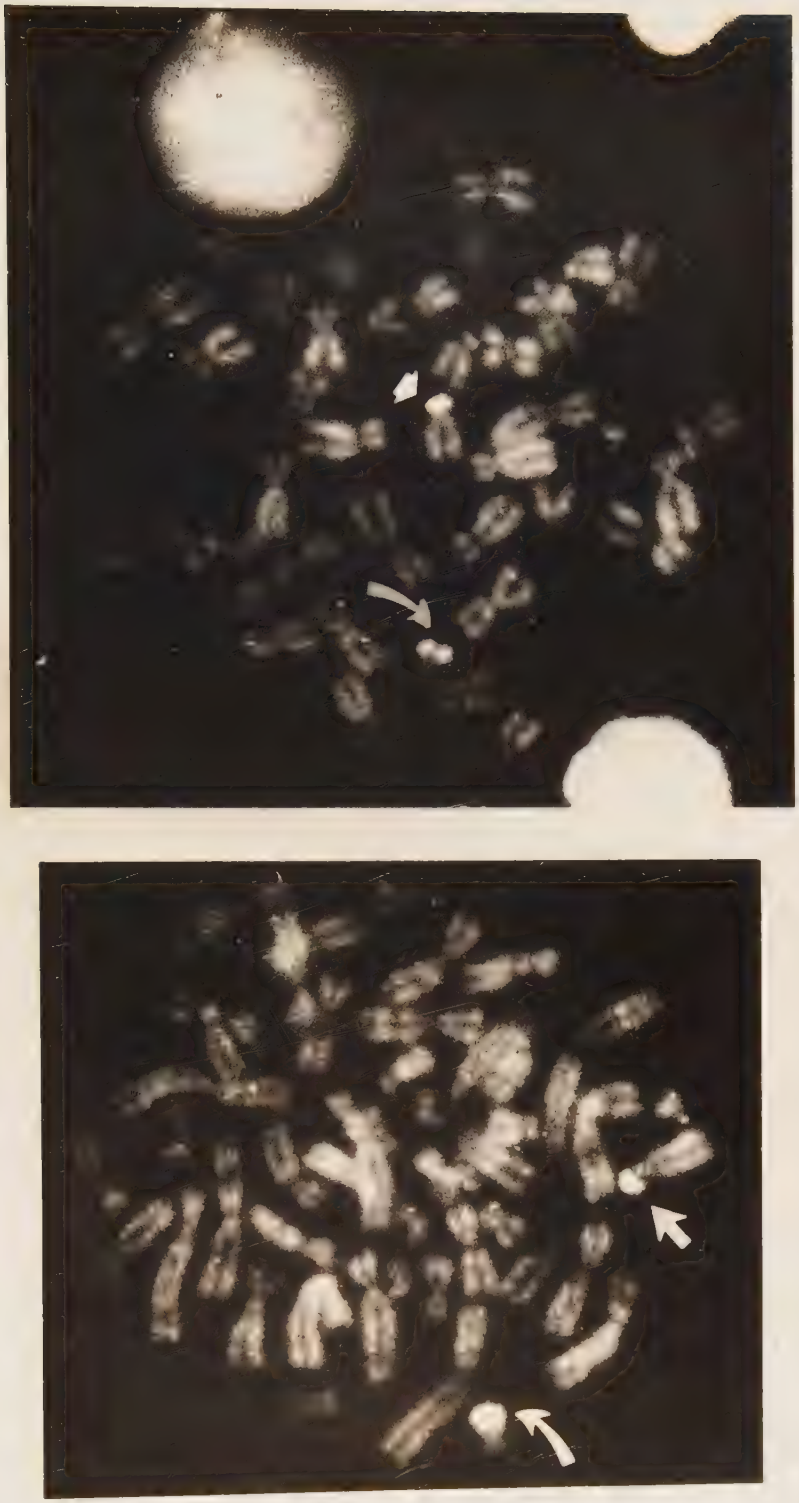

Figure 29 - Chromosomes from a male individual showing two intense regions after staining with quinacrine mustard: the normal Y chromosome and unusually large fluorescing satellites.

In one of the six males with double F-bodies in the lymphocytes, the chromosome analysis showed 47 XYY, and in another the presence of unusually large intense fluorescing satellites on one of the D-group chromosomes was seen. (Figure 29). The other 4 male babies are being followed-up.

Female-population.

Normal: Of the 986 females examined, 972 (98,5%) were classified as being normal on F-body counts of lymphocytes from cord blood. An average of 1 possible F-body in 30 cells of normal females was found.

Abnormal.

High F-body count: On examination of the first slide 1,4% of the 986 females were classified as having a high F-body count. Counts of repeated slides showed 2 of these to be normal. Of the remaining 12, 6 were re-examined and 4 were classified as normal on metaphase studies. A distinct fluorescent region on one of the autosomes was noted in each of these cases. The other two females were both classified as having a 46 XY chromosome complement. The remaining 6 are being followed-up.

DISCUSSION.

There is great variation in the size of Y chromosomes within the normal human population. The Y fluorescent body also varies considerably in size between different individuals but its size

and brightness was found to be uniform in all cells from the same individual.

Of the 1053 males examined 5% were found to have an abnormal F-body count after the examination of the first slide. 1% were found to be normal after reexamination of a blood smear from the same cord blood specimen. A few of the 4% males with a low F-body count, showed a Y chromosome with a very small fluorescent region, upon examination of the chromosomes. The incidence of Y chromosomes with a small intense fluorescent region or with no fluorescent region is still unknown because a large number of persons are still being followed-up.

The value of this technique is demonstrated in the identification of a 47 XYY chromosome constitution in a male with double F-bodies. The presence of unusually large intense fluorescing satellites on one of the D-group chromosomes resulted in the classification of the blood smears as having double F-bodies in another male baby.

The incidence of 2 females out of the 986 examined with a 46 XY chromosome complement is unexpected. Praeder (1957) estimates it as 1 in 20000 and Hauser (1963) believes that it occurs in 1 out of 2000 males. The discovery of many cases described in recent years in the literature, suggests that the incidence is definitely higher than previously estimated.

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Table 13: F-body counts in 600 lymphocytes from blood smears of 10 normal males and 10 normal females.

MALES			FEMALES		
	F-body in cells			F-body in cells	
No.	Present	Absent	No.	Present	Absent
1	19	11	11	1	29
2	15	15	12	0	30
3	11	19	13	0	30
4	13	17	14	0	30
5	25	5	15	2	28
6	19	11	16	1	29
7	20	10	17	0	30
8	25	5	18	1	29
9	25	5	19	0	30
10	21	9	20	2	28
Total	193	107	Total	7	293
F-bodies present 64% (± 6%)			F-bodies present 2,3% (± 2%)		

Table 14: F-body counts in 1000 buccal epithelial cells of 10 males and 10 females.

MALES			FEMALES		
	F-body in cells			F-body in cells	
No.	Present	Absent	No.	Present	Absent
1	30	20	11	2	46
2	39	11	12	3	47
3	25	25	13	3	47
4	31	19	14	5	45
5	35	15	15	10	40
6	38	12	16	5	45
7	32	18	17	4	46
8	31	19	18	3	47
9	35	15	19	6	44
10	37	13	20	5	45
Total	333	167	Total	46	454
F-bodies present 66% (± 5%)			F-bodies present 9,2% (± 3%)		

Table 15: F-bodies present in amniotic cells at 34-38 weeks of pregnancy.

MALES			FEMALES		
	F-bodies present			F-bodies present	
No.	Positive	Negative	No.	Positive	Negative
1	20	10	1	0	30
2	10	20	2	0	30
3	0	30	3	0	30
4	8	22	4	1	29
5	12	18	5	0	30
6	12	18	6	0	30
7	1	29	7	1	29
8	15	15	8	0	30
9	1	29	9	0	30
			10	3	27
			11	0	30
Total	79	191	Total	5	325
F-bodies present:	29%		F-bodies present:	1,6%	

C H A P T E R VI.

FLUORESCENCE OF THE Y CHROMOSOME IN METAPHASE.

Since Zech (1969) first reported that the normal human Y chromosome is easily recognized by its especially bright fluorescence, many studies have shown that it is the distal part of the long arm of the Y chromosome in metaphase, that fluoresces brightly after staining with certain fluorochromes. The presence of this region in interphase cells of normal males also was recognized by Pearson et al (1970) and George (1970), after staining with quinacrine dihidrochloride and quinacrine mustard.

It is well known that the normal Y chromosome exhibits polymorphism of the length of the long arms (Bishop et al (1962), Cohen et al (1966), Unnerus et al (1967). In the course of their study on fluorescence of the Y chromosome in various tissues and subjects, Retief and Van Niekerk (1971) found no intense fluorescence of a short Y chromosome in a phenotypically normal male who complained of infertility. Bobrow et al (1971) studied quinacrine dihidrochloride stained Y chromosomes of different lengths in 12 selected subjects, and showed the variation in length of the Y chromosome occurs only in the highly fluorescent region. These findings were confirmed by Robinson et al (1971) in their study of a few selected cases with variant Y chromosomes.

An investigation of the Y chromosomes in the normal male po-

pulation was undertaken to estimate the variation in lengths of the different regions of the Y chromosome.

Methods.

Blood cultures for chromosome analysis were set up from blood samples taken from 50 newborn male babies. The chromosome preparation was performed according to the method of Moorhead et al (1960). The air-dried slides were finally passed from absolute alcohol steps into Mac Ilvaines buffer solution at pH 6,8 stained for 10 minutes in a 50µgm/ml solution of quinacrine mustard and sealed in buffer.

The stained preparations were examined under a Zeiss fluorescence microscope with exiter filter BG12 and barrier filters 53/44. Suitable metaphase plates were photographed using either Agfa 50 B 65, Kodak Tri X or Panatomic X-films. Exposure times varied from 20-40 seconds. Photographs were printed on hard paper and enlarged approximately 3000X.

Measurements.

The non-fluorescent (NF) and the brightly fluorescent (F) regions of the Y chromosome, as well as the total length of a number 3 chromosome, were measured on each metaphase. The measurements were made in arbitrary units using a map measurer. The number 3 chromosome was chosen for a standardizing measure of chromosome size. It is easily recognizable chromosome and is also apparantly deviod of secondary constrictions, which could effect the individual lengths of the chromosome at a given state of contraction.

A total of 267 Y chromosomes and the corresponding no. 3 chromosomes were measured in 43 normal males. The length of the Y chromosome and the two regions NF and F were expressed as a proportion of the total length of the no. 3 chromosome.

RESULTS.

A negative correlation coefficient was calculated between the two regions NF and F, of the Y chromosome, indicating the independence of these two measurements.

In an analysis of variance of the non-fluorescent (NF) portion alone, no significant length variation was calculated between the 43 subjects ($F_{42,266} = 1,36$). For the brightly fluorescent region, F, however, a highly significant variation between subjects was measured with an F-value of 5,38 ($F_{,01}=1,69$). Therefore, virtually all differences in total length of the Y chromosome are accounted for by variation in the area of intense fluorescence.

A mean fluorescent index (F-index) of F/NF (fluorescent region divided by the dull fluorescent region) was calculated for each individual. These F-indexes varied from 0,73 to 1,48 with a mean of 0,90 for the 43 individuals studied.

The mean standardized length of the NF portion of the Y chromosome found in 267 cells of the 43 males, was calculated as $0,1917 \pm 0,002$.

In about 10% of cells of one male studied the Y chromosomes stained with double intense bands on the long arm. These Y chromosomes were of the same relative length than those seen with single bands.

DISCUSSION.

The Y chromosome in normal males can vary from the size of a G-group chromosome or smaller, (Borgaonker 1969), to one that is as long as a D-group chromosome. In a survey of normal males, it was found that the variation in length is accounted for only by the variation in the fluorescent (F) region of the Y chromosome.

The Y chromosomes in the present study varied from a length slightly bigger than G-group chromosomes to one comparable to an E-group chromosome. Exceptionally short or long Y chromosomes were not found in the 43 males studied.

The linearity of the relationship between total length and length of bright fluorescent region, was noted by Babrow et al (1971), and they inferred that this may persist to a point where an extremely small Y would have no fluorescent material at all. This length coincides with the mean length of the non-fluorescent portion of the Y chromosome. The length of the short chromosomes described as being non-fluorescent (Retief and Van Niekerk, 1971), was equal to the length of the non-fluorescing portion, and can be described as having the same size as the long arms of a G-group chromosome. Borgaonkar (1971) state that in a survey of 2000 boys they found that small Y chromosomes are not very common. Unnerus et al (1963) measured the Y chromosome when stained with conventional dyes, and found that the size has a normal distribution. On the basis of fluorescence studies however, it would seem reasonable to suppose that this size distribution of Y chromosomes is normal in the F-region only.

No specific phenotypic effect was noted in subjects with a large variation in Y chromosome size. Court Brown (1967) and

others suggested that the Y chromosome is inert in gene transcription. The role of the Y chromosome however, in testis formation and the induction of masculinization is generally accepted, with these functional genes situated near the centromere. In a survey of Y chromosome fluorescence in infertile males (unpublished), 3 males were detected as having short Y chromosomes, under-developed testes with azoospermia or low sperm count. Although the evidence is not conclusive, it seems that the Y chromosome can be assumed to be normal in functional length up to a size where the fluorescent portion is lost, beyond which, i.e. shorter than the long arm of a G-group chromosome, it should be classified being abnormal in length.

On the other end of the scale, Wilson et al (1971), Kim et al (1971) and Bühler et al (1971) reported on long Y chromosomes with double bands on the long arm. Except for a patient described by Kim et al (1971) all six patients described had a long Y chromosome as large as D-group chromosomes. The possible explanation put forward by them was that the fluorescence pattern of long Y chromosomes shows variants of different kinds, or that these cases represent examples of structural rearrangement such as duplication of a portion of the long arm material or insertion of autosomal or X chromosomal material.

In the present survey of 43 normal males, a Y chromosome was detected with polymorphism in the banding pattern in the same subject. In about 10% of cells examined, a Y chromosome with two bands on the long arm was seen. These chromosomes had the same overall length as those with one band. The F-indexes of the double banding Y's measure 1,0 against a mean of 0,90 of one-band Y

chromosomes - a non-significant difference. The possibility that all Y chromosomes in this subject have double bands was excluded by examining the fluorescence of late prophase and early metaphase figures, where only one band was recognized on the long arms.

With this evidence of a mosaic for one-band and two-band Y chromosomes of the same length, it seems that the occurrence of double bands on the Y chromosome is due to a polymorphic condition rather than a structural rearrangement. The correlation with long Y chromosomes could be explained by the fact that in males with a high F-index, higher than 1,0 - this polymorphism would be more easily recognized.

The observation of the duplex - form of F-bodies infrequently in interphase cells of certain normal males, can be accounted for by the presence of these double-banded Y chromosomes. An investigation of Y chromosomes in subjects with a relatively high incidence of duplex F-bodies is required.

A method of description of the length of the fluorescent portion of different Y chromosomes was developed by expressing the length measurement of the fluorescent region as a proportion of the more stable non-fluorescing region. (F/NF). In a series of 43 normal males, a mean F-index of 0,90 was measured in 267 Y chromosomes. A positive correlation between the length of the Y chromosome and the size of the F-body was noted by several authors. In a survey of 2039 newborns, for the presence of F-bodies, the same correlation was observed. In males with a very low F-index, the F-body was small in interphase cells and sometimes led to incorrect sex-classifications.

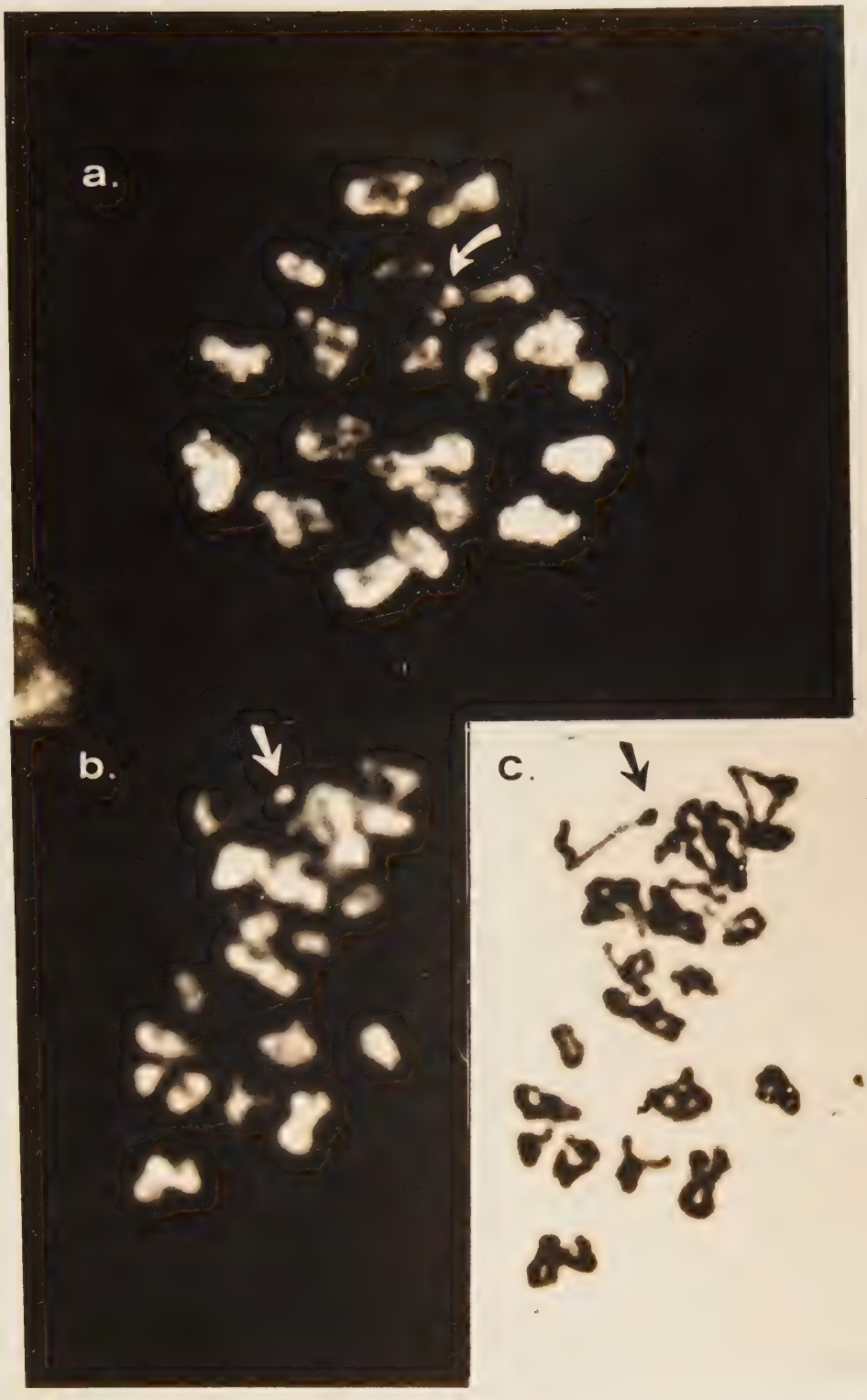

Figure 30 - The intense Y chromosome fluorescence seen the X-Y bivalent of:

(a) a male with a short Y chromosome and

(b) a male with a normal sized Y chromosome.

(c) The bivalents in (b) is restained with Giemsa.

Y chromosome fluorescence in meiosis.

Testis tissue from two normal males were prepared for the study of meiotic chromosomes according to a modification of the method described by Yunis (1965). The slides were stained for Y chromosome fluorescence as described in the previous chapter. Cells in various stages of meiosis were examined for the presence of the Y chromosome.

Spermatocytes from an ejaculate specimen of an XYY male were stained and examined for the presence of F-bodies.

Results and discussion.

The Y chromosome was recognized in the sex vesicle in prophase (figure 28), as well as in the X-Y bivalents of metaphase I of meiosis. The association of the short arms of the Y chromosome with the X chromosome in the X-Y bivalent can be seen in figure 30(b) and (c). Figure 30a shows a short Y chromosome with a low F-index in association with the X chromosome.

The short or long arms of the Y chromosome in the X-Y bivalent cannot be identified after staining with conventional staining methods. (Figure 30c). Staining the preparation with quinacrine reveals the light fluorescent short arm in association with the X chromosome. These observations confirms the results of Pearson and Bobrow (1970), using quinacrine dihidrochloride as stain.

In the examination of the ejaculate specimen of the XYY male, only 3% of spermatozoa had two F-bodies, and 17% one F-body. The high percentage presumably X-bearing sperm (80%) is unexpected. A bias, however is introduced into the results. The spermatozoa without F-bodies are believed to be X-bearing and only the positive F-body spermatozoa are counted in a specimen.

C H A P T E R VII.

SUMMARY AND CONCLUSIONS.

Summary.

Normal human chromosomes stained with quinacrine mustard could be visually classified into 24 classes, combining the chromosome classification method based on size and centromere position with that of the fluorescent banding patterns.

A photo-electric method of recording wave patterns of the bands on the chromosomes was found useful in the identification, and important in the description of the fluorescent intensities of the bands.

An attempt was made to unbiasedly select a series of chromosomes to be used as a range of normal chromosomes in an automated recognition program. The average patterns calculated from the mean intensity measurements of the normal chromosomes were tested for similarity in a computer program AVTEST. Comparing the chromosome patterns within the length groups A, B, C, D, no. 16 and E, the pairs of patterns 6-8, 7-X, X-10 and 14-15 were found to overlap. All other patterns were found to differ highly significantly, measured in all combinations against the other patterns within the groups.

A computer program CROTES was devised to identify a normal

chromosome pattern of numbers 1-18 and X chromosome, utilizing intensity measurements alone. The intensity measurements can be performed by computer and with minor interaction by a technician the computer system can identify all chromosomes of numbers 1-18 and X. The mathematical number assignment is done by computer. Of the 285 patterns tested 91% were classified correctly under one of the 19 chromosome numbers. The identification of the small chromosomes in groups F and G were found to be easy and depended only on an ability to distinguish between bright positive and light positive fluorescence. Studying the fluorescent intensities in these chromosomes therefore yielded little information.

All the chromosome patterns except numbers 8, X and 14 were found to differ highly significantly from the other patterns in the karyotype. The sequence of identification in the automated program was found to be important. Identifying the C-group chromosomes in strictly numerical order with the X chromosomes as the last to be identified, resulted in the correct classification of the chromosomes. The no. 7 chromosome was found to differ significantly from the mean X chromosome pattern, but comparing the X chromosomes with the mean no. 7 chromosome pattern yielded no significant differences. The same was found when comparing chromosomes 6 and 8, and 14 and 15 with the patterns of numbers 8 and 14 overlapping.

The fluorescence of Y chromosomes in interphase cells was studied in normal individuals as well as in individuals with abnormal Y chromosomes. The results were used as a yardstick for a survey for Y chromosome abnormalities in the newborn population. Studying lymphocytes in blood smears for the presence of F-bodies

was found to be an easy and reliable method. F-bodies were counted in 64% of lymphocytes of normal males, and in 2% of normal females. In an XX-male the counts were classified as female, while an XYY-male gave a high percentage of double F-bodies in lymphocytes. In a male with a Y chromosome shorter than a G-group chromosome, no typical fluorescence was seen in either interphase or metaphase cells. The results were interpreted as being of female origin. In two females with an XY chromosome complement a high F-body count, similar to that found in males, was made.

The presence of F-bodies were studied in amniotic cells from specimens taken late in pregnancy. The correlation of the results with the sex of the babies at birth revealed that in 3 preparations of the 20 examined the results were interpreted incorrectly. An analysis of F-bodies in amniotic cells taken at earlier gestational ages is required.

A survey of the newborn population was undertaken to determine the incidence of abnormalities of Y chromosome fluorescence through an examination of F-bodies in lymphocytes. This survey is still in progress and because of the migrant population that is being studied, the follow-up of babies found to have abnormalities, is not complete. Preliminary results revealed that 95% of males born in Tygerberg Hospital were classified as having normal F-body fluorescence, based on the examination of the first slide. In 98,5% of females studied the results were interpreted as normal. Of the 5% males classified as abnormal on the first slide 1% were found to be normal upon re-examination of a blood smear of the same cord blood specimen. Of the remaining, a high percentage is still under examination. One baby (1/1053) with double F-bodies in blood

smears was found to have a 47 XYY complement. A male baby with unusually large fluorescent no. 13 chromosome satellites have also been shown to have two F-bodies present in the lymphocytes. Four males with double F-bodies are being followed-up. In 24 males with a low F-body count out of a total of 1050 studies, 5 have subsequently been shown to have a Y chromosome with a very small fluorescent region; 19 are still to be followed-up.

In the examination of the female babies 1,5% were classified as having an abnormally high F-body count after the examination of the first slide. In counts of repeated slides 2 of the 14 were classified as normal. Of the remaining 12, 6 were re-examined and 4 were classified as normal on metaphase studies with a distinct fluorescent region noticable on one of the autosomes. Two females were classified as having a 46 XY chromosome complement and the remaining 6 are being followed-up. This incidence of 2 out of a total of 986 46 XY females, and the possibility of a higher occurrence, is unexpected.

The Y chromosome fluorescence was studied in mitotic metaphase of 43 normal males and in meiosis metaphase of two individuals. When measuring the fluorescent behaviour of Y chromosomes in metaphase in normal males, it was found that the variation in length is accounted for only by the variation in the fluorescent region. The formulation of the F-index was found neccessary to describe the fluorescent portion of a Y chromosome in terms of the stable non-fluorescent portion.

In the meiotic studies the intense fluorescent portion of the Y chromosome could be seen in prophase as well as in short arm association with the X chromosome in the X-Y bivalent.

Conclusion.

For 10 years since the first reports of a useful method for counting and identifying human chromosomes, the chromosomes were classified into 11 groups. Only chromosomes numbers 1, 2, 3 and 16 and sometimes Y chromosome could be identified with certainty.

With the discovery of banding patterns on chromosomes after staining with certain fluorochromes or after denaturing pretreatments, identification of all the individual chromosomes is possible. The application of the quinacrine mustard staining technique was proved to be a reliable method for the individual identification of normal human chromosomes.

The application of the fluorescence technique has far reaching implications in Human Cytogenetics. A method that could quantitatively describe the patterns of fluorescence was found to be essential and certainly superior to the subjective methods used previously. A photo-electric method of obtaining a wave pattern not only describes the location of the bands accurately but also the relative intensity of the bands. Recently Francke(1972) applied this technique to 16 individuals carrying various types of translocations in balanced and unbalanced states, and reported the interesting finding that no evidence was found of translocations being reciprocal.

It is of both theoretical and practical importance that chromosomes involved in translocations should be identified accurately, and if possible the breakpoints defined. Some chromosomes might be predisposed to form translocations and special mechanism of

origin have then to be considered. The gametic output might be different depending on the chromosomes involved in translocations. These observations are obviously of practical importance in genetic counselling.

The use of the characteristic quinacrine fluorescence patterns to identify human chromosomes in man-mouse hybrid cells, was demonstrated by Miller et al (1971). In their studies the human chromosome retained in a hybrid alone derived from human cells and a mouse line deficient in thymidine kinase, has the fluorescence pattern of chromosome no. 17. The use, therefore, of this technique in hybrid studies should greatly enhance the value of these hybrids for linkage studies. Nesbitt and Francke (1971) showed that the cytological localization of all linkage groups in the mouse should now be possible through the use of quinacrine mustard staining for the identification of mouse chromosomes.

In clinical cytogenetics a vast amount of material has to be examined, yielding a relatively small number of interesting cases. A system for the recording of fluorescence patterns and the measuring of relative intensities of the bands, suitable for computerized analysis of large numbers of chromosomes, has been developed. The use and application of an accurate automated system for the recognition of human chromosomes in the cytogenetic laboratory therefore means that not only could the growing volume of clinical work be dealt with, but the interest in research be stimulated to expand.

The discovery of Zech (1969) of the presence of the Y chromosome as a bright spot in interphase cells and its application increases the value of nuclear sexing and complements the Barr-body

test. Male populations can now be screened for the presence of abnormalities in the Y chromosome. The discovery of a higher incidence than was expected of 46 XY females born at Tygerberg Hospital, as well as an 47 XYY male, proves that the application of this technique is reliable. The intense fluorescence of the distal portion of the Y-chromosome was shown to be distinguishable in all stages of the cell cycle in meiosis as well as in mitosis. Bobrow et al (1971) have used this technique to demonstrate that the Y chromosome is spatially associated with the nucleolus. Utilizing this phenomenon of tracing the intense fluorescent regions through various cell, stages might reveal the underlying mechanisms of association in satellited human chromosomes. These are believed to be the nucleolus-organizing regions in the cell.

The variation in length of the Y chromosome in the normal population has been recognized since the earliest studies of the human chromosomes. Staining the Y chromosome with quinacrine mustard and measuring the intense and light fluorescent regions revealed that the variation in length of the human Y chromosome is due to a structural loss of the fluorescent portion of the Y chromosome only. The need to describe the Y chromosome in terms of its fluorescent staining abilities was realized, and the F-index, the fluorescent region, measured as a proportion of the stable non-fluorescent region, was formulated. From studies of the Y chromosome interphase fluorescence, a correlation was observed in the size of F-bodies and the F-index of the patient. A small F-body was found in individuals with a low F-index. Y chromosomes shorter than the long arm of a G-group chromosome show no intense fluorescence with an F-index of nil. An investigation of short Y chromosomes, identified through non-fluorescence in interphase cells,

might reveal the Y chromosome to be normal in functional length only to the size where the fluorescent portion is lost. Shorter Y chromosomes may be associated with abnormal testicular function.

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APPENDIX 1:

Standardized intensity measurements of chromosome No. 1.

Number of different No. 1 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
40	53	52	33	42	48	45	60	85	52	81	77	48	94	59
55	100	84	48	83	72	63	83	103	82	103	125	89	124	81
87	110	102	73	100	86	84	86	116	97	111	95	100	121	101
91	123	99	125	121	124	115	95	143	123	128	93	116	109	126
103	133	107	143	146	114	133	126	143	134	137	125	129	112	110
103	146	134	139	142	110	137	109	89	123	124	132	141	106	101
99	133	125	125	146	95	130	97	89	112	103	111	120	100	91
87	120	105	114	104	76	91	89	62	97	86	84	98	76	91
91	93	105	84	63	52	45	89	45	59	56	45	75	73	75
79	50	84	51	17	48	35	49	40	48	34	41	57	57	73
99	50	58	44	42	48	42	57	53	59	38	41	75	54	101
103	56	93	59	79	62	87	103	98	108	81	77	104	73	118
131	123	134	92	112	138	105	152	143	130	94	132	127	124	122
143	156	145	121	121	162	130	158	138	149	116	152	148	148	142
147	150	151	136	142	177	144	169	143	156	133	152	136	139	136
159	123	134	176	154	162	158	152	138	130	167	129	123	136	136
123	96	99	128	142	129	147	115	120	112	141	123	111	124	118
111	80	84	121	125	138	140	92	103	97	103	104	82	97	97
83	66	64	110	92	95	119	77	94	74	81	98	75	82	71
63	37	41	77	29	62	49	40	53	56	61	61	45	51	49

APPENDIX 2:

Standardized intensity measurements of chromosome No. 2.

Number of different No. 2 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
36	50	47	43	53	52	52	52	48	63	71	60	51	58	50
67	97	81	71	93	87	108	72	90	81	95	110	81	106	88
98	95	76	86	88	114	132	97	115	98	116	122	96	118	106
112	111	91	102	105	121	115	132	121	98	128	119	112	110	119
124	116	101	105	113	114	101	135	105	98	119	122	102	125	115
130	116	99	93	123	108	101	126	96	102	125	102	92	108	108
122	97	103	93	103	94	80	109	115	98	101	110	69	108	95
88	71	81	83	63	69	49	52	91	56	92	72	57	77	56
83	79	76	86	45	42	56	57	54	93	48	65	77	56	54
93	84	101	108	80	76	97	66	75	93	80	85	110	77	78
101	89	121	105	108	104	101	66	87	88	98	90	116	91	101
119	103	116	99	125	111	104	109	93	110	110	92	120	100	110
119	124	133	111	120	132	101	120	124	120	119	107	134	127	132
122	121	133	139	138	114	118	135	136	127	137	117	138	127	129
117	116	126	151	118	114	132	126	145	122	107	119	130	127	123
130	134	138	145	125	146	111	140	121	139	83	117	130	104	119
117	132	131	133	143	135	139	140	112	132	122	122	128	114	119
114	121	118	111	113	132	122	123	112	117	101	115	106	100	121
78	92	94	93	90	97	118	100	100	105	92	92	100	112	115
28	50	32	40	55	35	63	43	60	61	53	62	49	54	60

APPENDIX 3:

Standardized intensity measurements of chromosome No. 3.

Number of different No. 3 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
60	26	43	55	49	55	50	53	44	73	59	36	45	42	28
89	80	79	113	85	104	69	114	85	101	110	78	80	127	107
109	109	115	124	121	138	130	138	105	117	118	121	125	133	113
109	127	108	111	150	136	144	138	113	121	118	135	135	118	135
105	135	115	98	118	104	122	110	113	117	95	146	122	100	119
97	124	93	103	98	89	111	99	97	101	79	110	112	81	110
109	102	101	98	92	86	116	106	101	85	87	93	100	84	86
133	109	115	108	92	98	139	110	117	97	99	82	115	102	92
157	138	115	124	111	112	133	135	129	117	138	121	125	139	101
145	157	176	148	144	187	175	185	169	177	177	157	151	142	135
153	124	161	148	177	150	136	128	149	173	122	175	154	189	171
105	113	118	127	160	112	100	110	113	113	99	118	125	102	119
113	120	108	106	134	118	83	92	125	81	102	103	115	124	104
93	98	97	82	88	110	75	75	105	77	87	89	96	93	87
81	87	90	82	65	92	69	57	93	73	87	75	77	75	80
77	84	82	69	52	72	83	64	77	93	91	71	71	57	80
81	84	97	100	75	58	80	89	77	101	114	78	83	75	98
81	80	90	98	82	75	78	96	81	93	106	89	87	84	98
64	65	61	74	65	63	66	71	68	60	83	78	61	75	80
40	36	36	34	39	37	39	28	40	32	28	43	19	57	55

APPENDIX 4:

Standardized intensity measurements of chromosome No. 4.

Number of different No. 4 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
37	31	38	41	28	44	45	35	38	47	32	39	29	41	35
91	72	65	76	78	83	101	69	85	88	64	66	76	77	74
105	89	78	96	97	104	121	94	114	92	84	109	94	100	106
111	106	91	88	93	107	101	103	103	112	104	107	100	105	114
118	94	105	85	82	97	85	114	103	105	102	94	96	98	94
118	82	116	90	93	95	87	119	94	99	91	92	78	95	91
109	70	112	119	123	97	105	122	110	114	99	94	117	93	78
107	111	118	119	123	110	96	117	107	114	114	109	119	98	97
102	125	112	114	110	102	101	112	110	123	111	125	113	109	114
105	111	118	117	108	110	116	105	116	123	113	117	117	109	116
113	120	114	108	106	115	123	108	118	110	113	117	123	102	116
102	113	116	121	113	112	114	110	125	107	113	111	117	109	122
102	115	118	121	123	124	112	99	121	108	115	113	129	116	124
104	125	122	124	132	121	116	110	110	103	118	111	113	123	127
115	130	112	122	128	122	119	110	110	108	118	115	98	125	119
98	115	106	122	119	109	114	115	112	99	129	113	115	118	112
107	123	116	117	115	117	121	106	114	129	115	111	131	116	112
105	120	114	110	117	114	96	115	107	112	113	107	119	116	119
98	96	89	77	82	83	87	87	71	75	100	96	74	98	86
50	51	38	31	26	32	36	46	31	30	50	49	39	50	40

APPENDIX 5:

Standardized intensity measurements of chromosome No. 5.

Number of different No. 5 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
53	50	37	35	44	37	27	41	27	40	43	47	52	38	32
99	108	94	77	83	80	73	94	62	101	98	78	96	106	73
114	129	120	108	113	108	124	115	118	154	159	104	118	136	120
108	106	126	115	111	108	150	123	127	133	132	125	135	133	124
112	58	96	102	97	113	148	100	111	108	95	125	133	94	125
110	58	64	83	92	91	116	96	76	70	66	104	114	54	97
91	101	82	83	94	115	73	76	71	87	73	73	89	87	59
73	114	141	113	111	126	97	100	105	110	96	88	94	106	90
101	127	137	111	120	130	107	112	116	126	121	107	108	118	111
124	132	124	125	118	139	116	125	123	133	114	117	108	140	122
116	110	118	127	115	136	133	149	145	145	130	128	114	143	125
112	134	103	125	127	130	136	135	140	126	139	140	121	118	134
116	129	84	131	129	115	121	129	136	124	130	148	125	120	125
110	121	97	119	122	97	111	117	125	122	121	133	116	122	122
107	114	116	113	106	100	90	98	98	103	98	101	108	108	129
101	108	103	108	97	97	90	96	98	82	82	91	89	94	98
93	103	101	92	94	97	92	106	111	80	84	96	104	87	90
101	101	105	88	104	84	94	94	107	75	89	91	75	92	100
95	65	109	88	83	58	73	67	69	54	84	62	56	64	81
61	30	41	54	39	37	29	24	33	26	45	39	42	37	45

APPENDIX 6:

Standardized intensity measurements of chromosome No. 6.

Number of different No. 6 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
37	43	40	55	43	55	39	56	49	38	32	38	43	45	41
67	86	61	79	72	85	91	84	79	66	72	76	74	74	72
94	105	52	87	83	99	100	86	76	76	81	87	80	72	76
94	86	54	69	77	95	102	82	70	71	87	96	78	59	76
90	86	58	81	79	79	78	80	67	81	110	101	82	64	82
112	103	79	73	90	83	93	104	76	93	104	69	105	94	100
94	77	79	71	86	81	106	114	61	95	81	60	85	68	106
95	53	71	100	74	76	87	90	49	86	78	51	85	72	86
103	107	101	126	86	79	83	102	88	95	107	76	116	113	96
118	118	109	130	113	129	109	130	110	135	147	80	116	111	131
118	114	123	118	126	129	113	118	131	138	150	107	134	102	125
131	120	131	128	137	125	122	132	146	147	133	134	128	113	125
139	131	127	130	113	127	126	128	155	140	121	145	132	113	131
122	120	119	126	110	125	130	130	152	131	130	132	118	128	127
120	126	121	114	137	125	128	128	149	135	112	136	126	134	127
129	131	119	126	144	127	124	122	155	126	110	143	124	134	119
116	133	127	122	133	125	126	116	134	114	115	138	115	145	121
99	122	135	122	135	122	111	98	116	105	92	132	105	147	117
81	96	141	92	104	83	89	70	91	81	78	123	99	134	90
41	43	149	51	56	48	43	30	43	45	61	74	54	74	51

APPENDIX 7:

Standardized intensity measurements of chromosome No. 7.

Number of different No. 7 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
55	42	57	58	56	45	63	68	47	56	46	32	43	41	41
93	106	101	139	123	76	106	131	103	101	120	86	104	107	99
126	127	108	155	152	102	118	133	130	112	145	116	133	105	105
113	135	96	128	144	109	118	133	125	114	137	110	112	109	86
121	127	104	111	123	119	108	135	118	119	120	107	93	98	92
119	119	86	108	128	104	108	119	120	117	87	99	103	100	103
121	93	64	97	93	89	94	111	111	115	53	97	106	96	90
88	66	67	53	59	76	72	72	89	80	56	71	104	68	73
76	37	64	33	69	76	91	74	78	93	76	79	66	59	82
98	53	96	66	96	91	111	109	89	108	82	105	71	86	94
101	109	113	103	96	106	118	135	108	115	102	131	93	91	125
129	109	145	122	157	126	123	123	113	124	145	140	120	148	120
121	130	138	136	101	141	113	86	109	112	130	114	123	125	101
106	111	121	89	91	130	120	98	108	110	99	107	117	114	137
111	114	109	111	112	113	123	123	115	115	112	125	109	127	155
118	151	116	125	117	115	115	103	123	107	132	127	120	148	137
99	130	113	122	91	128	96	88	101	93	120	112	112	127	120
84	114	106	103	85	113	96	76	89	96	97	103	112	116	116
79	85	136	89	67	89	70	55	82	75	87	97	96	100	73
41	40	57	53	40	48	36	25	42	37	53	43	61	55	49

APPENDIX 8:

Standardized intensity measurements of chromosome No. 8.

Number of different No. 8 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
36	31	31	46	39	42	35	33	28	23	32	43	28	54	26
61	57	62	74	76	60	75	56	63	50	65	79	57	72	57
88	78	83	79	71	78	82	74	76	65	78	88	75	74	76
92	90	86	67	71	84	79	84	86	75	87	88	94	84	98
90	94	99	76	68	90	82	100	89	88	93	96	97	84	100
92	97	91	72	68	102	75	100	91	92	93	90	86	97	94
70	88	88	65	80	90	91	89	84	84	78	79	83	86	88
101	111	88	83	85	78	110	94	79	63	91	102	72	76	82
115	125	94	116	119	105	126	120	91	86	106	120	70	86	80
126	140	112	118	142	129	114	133	124	117	122	126	94	107	112
137	117	143	137	146	135	131	133	140	132	122	128	114	128	141
126	127	148	130	142	153	126	128	129	146	130	137	138	134	131
142	115	135	158	151	132	135	135	132	146	139	134	145	148	133
133	127	133	139	144	120	126	133	135	142	135	126	143	142	141
128	115	133	144	146	126	131	130	137	144	143	124	138	126	139
128	129	133	132	146	114	131	133	140	152	143	126	141	119	143
122	129	120	125	124	120	126	120	122	138	117	110	136	111	131
101	115	99	109	90	102	103	100	109	117	104	88	119	115	115
70	74	75	83	61	84	75	66	89	92	80	71	105	103	74
38	37	44	44	27	48	47	38	53	48	39	43	61	54	37

APPENDIX 9:

Standardized intensity measurements of chromosome No. 9.

Number of different No. 9 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
42	52	81	53	41	38	48	61	81	68	72	59	41	52	62
90	93	168	111	118	136	139	91	140	128	120	114	94	83	112
127	158	177	144	149	139	162	138	148	162	141	147	137	121	146
142	166	171	156	154	155	182	149	146	156	135	142	140	150	160
127	169	146	151	152	155	139	165	146	139	120	147	147	167	126
121	149	87	146	133	150	128	163	135	136	112	135	133	145	93
89	120	44	136	116	112	133	132	84	99	82	95	101	110	59
76	82	47	101	70	101	111	91	62	74	72	50	80	69	59
66	55	44	60	41	57	74	66	54	60	61	45	60	69	39
84	55	44	43	41	27	48	50	76	51	53	69	73	71	48
98	52	68	43	60	57	54	58	111	79	58	92	66	74	62
124	87	103	73	106	95	91	66	113	102	90	126	96	86	112
121	123	125	134	118	120	113	99	97	116	114	119	105	102	149
131	117	112	116	108	109	105	121	105	108	127	107	124	126	124
108	111	109	101	113	120	85	135	113	122	120	116	128	110	121
129	93	112	116	123	128	94	99	116	122	122	126	115	105	126
121	99	118	118	128	101	96	105	92	108	133	114	110	117	132
116	93	127	98	108	87	96	105	81	85	122	95	105	105	115
71	73	84	71	87	76	71	72	59	54	96	73	89	86	95
32	50	31	28	31	33	31	33	38	28	48	28	53	50	59

APPENDIX 10:

Standardised intensity measurements of chromosome No. 10.

Number of different No. 10 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
39	48	43	47	57	63	51	61	40	42	53	53	38	36	52
80	89	81	94	89	89	94	83	83	86	95	77	81	70	91
102	100	123	106	119	100	121	94	102	107	121	94	108	99	112
105	118	136	115	128	106	132	86	115	109	130	101	116	102	120
105	115	132	126	109	111	121	99	119	111	125	96	119	104	118
104	96	109	111	92	85	113	89	106	96	114	90	106	107	112
90	70	85	89	68	85	83	77	92	78	99	70	79	104	108
115	92	111	100	85	117	94	99	89	82	99	90	92	109	102
133	118	132	121	98	134	118	122	117	105	117	123	110	117	114
131	133	132	130	126	124	135	144	141	130	143	131	136	138	127
117	128	111	123	132	115	129	138	146	132	136	138	128	138	127
115	131	109	111	113	126	108	116	127	130	117	134	110	130	118
109	124	119	113	109	124	110	119	112	128	114	127	112	112	112
111	128	113	117	107	128	110	116	104	130	108	118	110	120	110
115	115	102	104	113	124	113	111	98	128	99	123	112	112	100
113	107	109	100	111	126	102	113	110	122	97	123	114	102	95
111	100	96	96	115	104	94	116	104	109	88	103	117	99	89
96	85	77	89	107	74	83	102	94	86	70	101	97	91	83
70	67	57	68	79	41	54	69	65	63	48	70	77	68	69
35	33	23	38	38	26	30	41	33	25	24	37	35	36	37

APPENDIX 11:

Standardized intensity measurements of chromosome No. 11.

Number of different No. 11 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
39	40	27	32	48	22	46	42	29	20	37	37	38	33	42
74	93	64	89	88	59	62	92	65	49	78	78	91	74	84
113	136	96	124	91	86	97	128	102	101	112	113	106	116	124
121	150	108	143	117	131	117	148	130	123	141	139	154	143	147
134	147	117	148	142	127	124	148	141	130	150	147	159	146	124
118	139	122	135	120	149	125	136	133	123	132	125	147	132	144
124	82	115	113	108	119	104	121	109	110	101	113	137	102	122
97	57	89	81	65	90	86	88	78	64	52	88	108	66	89
58	85	102	89	60	86	88	75	89	64	52	73	70	41	50
68	82	82	94	68	80	71	75	83	84	60	81	70	63	60
76	99	89	67	60	76	77	59	81	64	63	71	67	61	62
68	91	133	70	77	94	117	81	107	84	104	86	72	88	75
116	105	139	105	120	133	135	99	123	115	135	115	108	105	94
137	127	139	116	140	133	146	114	133	133	155	130	118	129	129
147	136	140	143	148	149	141	123	138	164	152	142	135	157	134
161	130	122	143	157	137	117	139	125	157	147	130	125	154	134
124	102	109	113	128	127	108	125	112	142	115	120	108	138	129
100	96	104	84	120	100	97	95	91	125	92	91	79	127	104
71	68	67	65	85	69	86	79	70	98	75	73	65	85	92
53	34	33	43	57	33	53	31	57	49	46	49	41	38	57

APPENDIX 12:

Standardized intensity measurements of chromosome No. 12.

Number of different No. 12 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
30	24	31	30	56	48	35	43	44	26	37	47	43	53	40
75	65	67	65	71	78	81	72	90	55	66	82	80	92	78
109	101	104	101	97	103	123	113	101	92	103	100	100	108	98
126	122	107	117	112	116	137	131	108	103	119	102	107	108	112
116	114	101	117	116	103	122	128	110	101	116	90	98	86	112
94	97	90	103	110	81	107	101	84	88	100	86	85	86	102
106	101	87	93	107	71	79	106	110	92	82	121	102	102	96
108	110	107	101	116	94	100	119	108	105	95	104	109	89	110
100	114	101	98	107	94	114	104	81	101	105	84	105	82	106
108	103	104	101	107	110	100	117	95	101	95	106	110	112	104
128	110	98	117	114	103	120	128	110	123	111	125	121	128	120
126	131	115	133	131	129	136	131	114	145	134	137	128	145	132
128	148	132	142	135	142	134	140	132	149	147	147	130	164	136
124	146	138	136	135	152	130	149	138	153	150	137	134	138	136
116	144	143	128	118	139	122	117	136	142	137	121	128	122	130
110	127	129	117	101	126	116	92	125	125	111	113	110	112	112
94	97	124	95	92	103	93	75	103	110	92	100	105	89	98
92	69	104	84	77	91	63	57	92	90	82	92	93	82	74
71	45	79	79	62	65	56	45	70	59	69	70	78	62	66
37	30	36	41	32	48	28	31	48	39	50	37	32	39	36

APPENDIX 13:

Standardized intensity measurements of chromosome No. 13.

Number of different No. 13 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
31	23	41	32	25	37	32	43	36	36	30	22	37	26	38
52	46	59	53	60	65	81	65	48	48	57	46	62	48	54
73	70	70	69	70	76	100	80	60	64	75	71	75	70	82
84	95	88	85	83	73	108	82	81	69	87	97	89	89	94
94	104	92	96	93	86	84	89	95	76	91	106	98	96	96
96	120	88	104	103	92	86	92	93	78	97	112	104	94	98
102	100	101	108	96	99	75	101	107	82	103	110	114	96	96
115	104	124	110	108	105	78	111	114	96	111	115	102	100	96
123	125	117	120	108	131	108	111	122	117	123	121	114	115	108
125	134	124	128	123	133	113	123	124	128	133	134	116	122	118
125	139	126	134	126	149	121	140	129	137	139	136	125	133	132
132	144	124	140	126	146	119	138	134	140	133	134	129	142	130
138	137	131	140	126	144	135	133	136	142	127	136	125	142	134
134	132	135	136	133	131	140	126	129	144	125	130	131	135	132
136	125	137	118	138	136	151	138	131	144	125	119	133	133	138
121	118	128	118	138	115	146	123	126	142	123	115	137	102	132
109	107	119	101	121	112	127	106	126	128	119	112	116	113	118
96	86	99	101	106	78	92	87	105	110	99	86	87	107	92
75	60	65	69	73	47	65	70	74	80	67	59	62	89	74
38	30	34	34	40	42	38	41	29	37	38	37	41	46	42

APPENDIX 14:

Standardized intensity measurements of chromosome No. 14.

Number of different No. 14 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
29	47	37	42	31	43	46	42	39	32	47	39	47	33	49
64	58	66	56	53	62	64	75	55	66	75	69	68	78	66
78	75	82	69	78	80	87	93	69	90	98	86	92	95	86
91	86	100	91	104	111	100	96	81	108	103	95	104	107	111
98	112	131	96	123	120	107	105	108	118	112	108	113	119	122
123	128	126	116	133	124	116	142	122	129	124	116	123	121	133
128	126	134	133	141	136	128	133	129	140	129	125	132	127	135
135	124	129	145	141	133	135	133	134	138	127	134	132	134	131
140	122	134	155	143	142	130	124	143	140	122	142	128	136	126
125	140	124	135	121	127	114	115	129	131	117	132	118	132	126
115	140	118	123	110	127	114	117	120	127	105	134	111	132	113
110	128	108	108	108	124	114	112	115	108	103	119	111	107	100
106	103	100	111	92	96	112	99	106	104	108	101	113	99	102
115	91	97	118	64	77	94	105	108	104	131	103	113	107	124
130	98	113	130	112	93	98	105	118	122	117	108	116	115	124
130	108	110	125	123	102	121	102	122	90	112	106	118	95	104
108	110	100	98	110	111	119	96	102	86	91	97	102	95	88
76	89	84	71	98	80	91	90	83	72	77	86	76	74	75
59	68	71	49	55	65	68	69	65	59	61	65	52	53	49
37	44	34	27	29	46	39	42	48	34	37	34	28	41	29

APPENDIX 15:

Standardized intensity measurements of chromosome No. 15.

Number of different No. 15 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
54	45	50	57	40	44	36	44	36	32	34	61	60	58	39
83	68	96	79	69	65	61	61	64	69	45	80	101	75	68
98	99	110	79	88	89	78	88	76	106	71	102	109	81	89
122	111	123	118	98	94	87	115	95	122	92	113	121	100	97
142	117	135	116	101	107	95	128	115	128	111	125	131	113	118
142	128	160	125	120	116	109	133	115	130	127	133	107	123	123
139	142	160	131	123	124	124	130	119	149	143	158	131	134	123
142	131	151	122	136	131	124	115	146	146	150	138	137	131	126
132	115	133	129	139	145	143	103	143	144	150	133	153	131	139
117	133	130	133	131	145	145	123	136	130	143	116	143	136	147
100	137	128	127	133	136	148	123	143	120	140	111	153	131	131
103	124	112	122	120	131	131	125	129	112	124	100	111	117	126
100	113	94	110	107	119	112	113	131	109	121	89	93	108	105
98	92	75	102	96	114	109	108	119	96	114	89	87	102	105
98	90	73	100	96	99	109	98	110	85	111	89	87	100	108
93	90	69	86	96	90	112	86	95	85	92	89	87	98	102
68	79	57	78	85	70	85	86	67	74	66	69	68	79	79
51	58	41	63	77	58	65	83	55	45	53	69	52	58	42
32	34	27	35	48	39	39	51	36	32	32	58	34	31	29

APPENDIX 16:

Standardized intensity measurements of chromosome No. 16.

Number of different No. 16 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
38	41	36	28	43	28	51	35	37	44	33	51	44	47	58
71	67	62	77	69	51	68	62	65	90	59	82	65	77	82
92	97	88	101	106	89	82	85	102	110	80	105	88	105	110
112	129	111	115	112	102	96	93	114	125	90	118	91	105	121
139	163	129	121	112	107	104	93	121	125	85	115	94	102	119
139	100	127	119	109	132	102	90	114	104	75	103	85	97	110
124	94	122	111	95	107	99	75	95	70	69	92	73	77	112
100	91	114	97	60	96	90	75	90	62	59	80	62	72	95
91	79	96	87	57	84	90	75	84	68	67	87	62	72	78
74	76	88	99	60	89	93	85	83	82	95	92	79	94	84
77	91	80	93	49	91	110	113	88	88	100	103	85	111	93
97	117	85	89	89	104	135	130	100	118	124	118	103	127	101
118	144	101	113	123	119	127	153	125	147	116	128	117	138	110
148	159	129	142	152	160	135	158	134	155	160	141	147	138	132
148	144	163	146	184	155	147	150	137	145	185	134	164	144	138
130	144	147	136	164	155	135	145	146	143	185	126	182	144	129
109	135	122	115	149	122	119	140	141	125	155	123	153	130	116
86	88	96	105	126	94	93	118	114	98	129	97	150	116	97
77	59	65	69	77	74	73	80	77	58	88	66	100	66	69
29	38	39	32	60	41	48	45	30	40	46	33	56	36	45

APPENDIX 17:

Standardized intensity measurements of chromosome No. 17.

Number of different No. 17 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
58	38	50	47	40	50	41	44	42	33	33	44	34	45	38
89	60	75	51	73	79	69	69	77	61	59	65	58	76	58
98	76	86	77	102	116	104	94	116	79	108	102	92	96	86
92	95	90	90	146	116	1I4	102	129	89	115	126	100	108	84
92	92	79	109	146	96	79	96	134	86	105	126	115	106	78
72	95	79	103	121	83	61	83	113	81	95	98	123	101	76
77	95	79	94	77	83	51	69	90	69	85	84	92	86	86
100	63	93	92	58	89	71	61	74	79	89	70	100	93	101
98	82	86	105	95	96	94	63	67	92	98	77	102	101	124
112	88	86	111	99	103	94	80	81	102	105	98	110	108	127
95	120	72	115	95	106	94	91	97	117	102	116	97	108	119
121	114	104	105	58	116	104	116	95	119	95	100	113	108	127
146	123	119	130	62	122	132	140	95	132	128	98	118	118	132
155	142	140	143	113	139	160	173	102	145	141	123	131	129	137
152	158	158	143	150	149	168	165	120	158	157	128	144	131	142
146	161	172	138	142	139	163	151	134	158	144	142	131	126	137
126	155	165	130	150	109	153	138	139	140	135	140	129	126	122
83	133	129	101	132	93	119	116	129	109	108	121	97	108	104
57	76	86	69	91	73	86	88	99	86	59	93	73	76	78
34	35	47	45	47	43	51	61	65	64	36	47	39	45	42

APPENDIX 18:

Standardized intensity measurements of chromosome No. 18.

Number of different No. 18 chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
27	27	33	34	47	43	29	33	27	46	28	34	37	37	36
53	46	68	68	49	62	67	69	67	61	56	66	61	70	67
71	60	74	88	67	79	78	80	80	73	62	79	81	85	81
74	81	85	113	87	85	81	85	83	73	59	68	83	88	79
67	69	81	79	85	70	70	80	77	70	49	61	74	75	85
84	81	70	81	87	74	65	78	68	61	40	56	59	72	87
104	96	89	86	72	74	67	78	92	70	46	64	72	80	88
124	123	107	115	76	91	86	94	105	78	68	59	101	88	101
143	127	120	122	108	117	110	126	132	111	96	114	135	118	124
141	127	129	127	150	136	121	139	145	131	127	145	131	130	126
127	133	129	142	162	134	129	137	147	143	136	150	124	145	126
125	142	131	126	164	145	129	137	140	145	139	137	126	138	126
127	138	133	102	150	136	129	133	125	148	148	125	140	140	124
137	144	129	106	139	134	134	130	125	145	154	145	135	133	127
133	146	122	117	137	138	145	142	133	141	161	155	135	133	127
149	146	122	136	132	123	151	149	133	138	173	165	139	135	129
120	121	122	117	105	121	137	126	123	133	167	153	131	130	124
92	94	113	106	92	111	124	101	95	121	139	99	113	105	118
59	62	81	86	67	72	91	62	68	82	90	61	83	60	78
39	35	61	47	34	47	40	25	27	46	40	43	40	37	46

APPENDIX 19:

Standardized intensity measurements of chromosome No. X.

Number of different No. X chromosomes:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
34	50	49	45	40	69	55	50	55	32	24	52	29	38	50
80	88	80	76	86	105	69	86	90	82	54	102	79	75	106
108	99	110	94	108	118	91	99	100	110	106	120	108	105	127
118	121	129	106	123	123	107	112	115	127	125	120	106	115	135
124	112	121	115	121	115	107	110	115	136	119	115	108	110	117
110	104	98	106	117	91	102	112	102	119	121	105	112	105	106
106	104	87	98	101	86	96	97	97	103	106	95	112	100	108
98	86	103	108	108	98	80	102	85	91	95	83	92	103	95
90	88	122	127	110	113	107	115	95	88	78	72	94	112	90
110	108	124	127	128	110	118	123	97	106	95	70	104	115	117
122	114	117	112	123	120	124	121	107	119	117	85	117	119	124
118	127	119	110	112	123	124	128	120	133	125	115	129	117	126
114	136	115	112	110	120	121	115	120	134	134	130	125	122	122
116	125	114	121	117	113	118	121	117	123	125	135	100	122	117
114	121	103	112	108	118	116	110	117	104	123	125	121	122	109
108	121	110	108	101	115	124	110	107	99	119	120	117	121	100
106	108	107	119	97	101	110	108	105	93	108	117	106	107	81
102	88	80	100	90	74	99	91	105	80	102	107	100	91	72
76	67	63	65	64	56	83	63	97	73	84	87	90	63	61
38	30	45	35	33	32	47	24	55	39	39	42	50	35	38

APPENDIX 20.

Identification of fifteen No. 1 chromosomes by computer program CROTES.

F-values of patterns tested against the mean values of chromosomes 1,2 and 3.

	No. 1.	No. 2.	No. 3.
1.	1,10	1,81	11,22
2.	1,21	7,24	12,14
3.	0,99	4,02	9,49
4.	1,72	6,53	17,70
5.	1,18	5,55	16,46
6.	1,12	5,39	20,49
7.	1,12	4,58	19,49
8.	0,76	4,27	14,36
9.	1,22	3,98	13,29
10.	0.28	2,87	11,86
11.	1,19	5,20	14,48
12.	0,66	4,54	15,53
13.	0,48	2,79	9,24
14.	0,81	4,09	11,30
15.	0,85	1,25	7,68
MEAN F-VALUE:	0,90	4,27	13,65

APPENDIX 21.

Identification of fifteen No. 2 chromosomes by computer program CROTES.

F-values of patterns tested against the mean values of chromosomes Nos. 2, 3 and 1.

	No. 2.	No. 3.	No. 1.
1.	1,40	6,28	1,52
2.	0,29	7,51	1,11
3.	1,08	8,55	2,06
4.	1,27	9,57	1,52
5.	0,71	9,34	1,59
6.	0,89	9,59	1,79
7.	1,40	10,01	2,86
8.	1,32	11,75	1,00
9.	0,96	9,59	0,82
10.	0,71	8,91	1,68
11.	1,39	6,32	2,00
12.	0,87	6,73	1,44
13.	1,11	8,20	2,36
14.	0,84	8,06	1,27
15.	0,46	9,67	1,57
MEAN F-VALUE:	0,98	8,67	1,64

APPENDIX 22.

Identification of fifteen No. 3 chromosomes by computer program CROTES.

F-values of patterns tested against the mean values of chromosomes 3, 2 and 1.

	No. 3.	No. 2.	No. 1.
1.	0.89	9,57	8,10
2.	0.96	8,22	6,61
3.	0.49	8,27	7,78
4.	0.50	7,56	7,75
5.	1.45	12,52	10,48
6.	1,18	11,10	9,30
7.	1,31	13,04	10,04
8.	1,08	12,13	11,12
9.	0,59	8,34	7,32
10.	1,00	11,12	9,50
11.	1,51	7,46	7,59
12.	0,78	9,93	8,98
13.	0,49	8,93	8,19
14.	1,36	11,07	10,05
15.	1,12	7,05	7,07
MEAN F-VALUE:	0,98	9,75	8,65

APPENDIX 23.

Identification of fifteen No. 4 chromosomes by computer program CROTES.

F-values of patterns tested against the mean values of chromosomes 4, 5, 6 and 7.

	No. 4.	No. 5.	(No. 6.)	(No. 7.)
1.	1,72	1,82	3,54	1,38
2.	1,13	2,67	1,34	2,66
3.	0,76	2,19	1,79	2,41
4.	0,86	2,85	1,13	2,39
5.	1,22	3,21	1,71	2,89
6.	0,44	1,62	1,30	1,41
7.	1,45	1,68	1,88	1,22
8.	1,09	2,47	3,01	2,01
9.	0,67	1,15	1,81	1,38
10.	1,48	2,15	2,95	2,26
11.	0,66	2,77	1,43	2,09
12.	0,58	1,37	1,85	1,93
13.	1,26	2,62	2,12	2,81
14.	0,72	2,35	1,16	1,36
15.	0,76	1,31	1,14	1,60
MEAN F-VALUE:	0,98	2,15	1,87	1,99

APPENDIX 24.

Identification of fifteen No. 5 chromosomes by computer program CROTES

F-values of patterns tested against the mean values of chromosomes 5, 4, 6 and 7.

	No. 5.	No. 4.	(No. 6).	(No. 7).
1.	1,30	3,60	3,11	0,98
2.	1,35	4,75	3,66	3,70
3.	2,04	4,74	5,57	4,11
4.	0,52	2,74	2,28	2,04
5.	0,34	1,66	2,42	1,79
6.	1,13	5,63	4,32	3,26
7.	1,17	7,73	6,41	2,84
8.	0,49	5,07	3,16	2,23
9.	0,96	5,07	3,50	3,05
10.	1,17	10,10	6,93	3,91
11.	0,92	7,55	6,03	2,91
12.	0.80	5,79	3,45	1,90
13.	1.02	5,75	4,57	1,47
14.	0,69	7,27	5,41	3,51
15.	0,82	4,02	3,12	1,75
MEAN F-VALUE:	1.08	5,41	4,26	2,63

APPENDIX 25.

Identification of fifteen No. 6 chromosomes by computer program CROTES.

F-values of patterns tested against the mean values of chromosomes 6, 7, 8, 9 and X.

	No. 6.	No. 7.	No. 8.	No. 9.	No. X.
1.	0,70	1,53	1,43	5,73	2,06
2.	0,59	1,66	2,97	6,19	4,86
3.	1,93	11,13	12,94	18,03	22,14
4.	0,80	3,22	2,02	9,44	6,27
5.	0,65	2,56	2,26	7,79	7,26
6.	0,53	1,70	2,13	5,68	4,17
7.	0,74	1,23	2,80	4,63	2,91
8.	0,79	2,02	2,40	6,97	3,54
9.	1,96	4,49	2,91	10,57	14,50
10.	0,75	2,91	0,65	8,84	6,01
11.	1,50	2,71	2,06	7,94	3,46
12.	1,66	3,31	4,52	6,86	10,35
13.	0,56	2,16	1,04	8,03	4,15
14.	1,48	5,04	5,67	11,91	12,94
15.	0,59	2,29	1,25	7,64	4,25
MEAN F-VALUE:	1,02	3,19	3,14	8,41	7,25

APPENDIX 26.

Identification of fifteen No. 7 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 7, 6, 8, 9 and X.

	No. 7.	No. 6.	No. 8.	No. 9.	No. X.
1.	0,50	3,78	10,59	2,05	1,55
2.	1,22	5,78	12,54	1,50	5,06
3.	1,28	2,39	6,14	4,94	5,69
4.	1,25	8,98	20,52	2,16	6,49
5.	1,27	8,85	21,17	2,16	6,55
6.	0,74	1,52	3,75	2,91	1,78
7.	0,51	3,15	7,50	2,91	0,99
8.	1,95	9,04	18,38	3,03	4,71
9.	0,37	4,24	11,39	1,52	1,45
10.	0,57	3,47	8,40	2,89	0,94
11.	1,00	5,76	15,78	2,14	7,06
12.	0,59	1,78	5,10	3,56	2,05
13.	0,95	3,54	10,49	2,28	2,89
14.	0,91	2,73	6,08	3,52	4,89
15.	1,60	1,88	3,81	5,36	5,31
MEAN F-VALUE:	0,97	4,45	10,77	2,86	3,83

APPENDIX 27.

Identification of fifteen No. 8 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 9, 8, 7, 10, 11 and X.

	No. 8.	No. 7.	No. 6.	No. 9.	No. X.	No. 10.
1.	0,67	2,74	0,90	7,68	4,06	3,42
2.	1,36	3,17	1,41	8,53	3,44	3,33
3.	0,43	2,31	1,04	6,94	4,36	4,90
4.	1,32	3,92	1,05	10,32	9,59	7,47
5.	1,98	5,28	1,89	12,46	11,15	7,78
6.	0,97	2,27	0,92	7,70	4,41	4,01
7.	0,74	3,18	0,79	8,91	5,10	4,33
8.	0,49	2,92	1,13	8,63	3,62	3,61
9.	1,39	2,69	0,72	7,69	5,28	5,69
10.	1,45	4,02	2,09	9,67	9,80	10,74
11.	0,28	2,58	0,80	7,69	4,84	4,60
12.	1,08	2,13	0,85	7,48	2,87	1,94
13.	1,67	2,79	1,56	6,84	6,99	11,98
14.	1,22	2,16	0,68	7,17	5,22	7,19
15.	0,78	2,68	1,43	6,64	4,88	6,52
MEAN F-VALUE:	1,05	2,98	1,15	8,30	5,70	5,83

APPENDIX 28.

Identification of fifteen No. 9 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 9, 8, 7, 10, 11 and X.

	No. 9.	No. 8.	No. 7.	No. 10.	No. 11.	No. X.
1.	1,16	10,43	0,82	7,46	1,84	2,64
2.	0,75	32,14	4,67	21,13	3,46	13,09
3.	1,77	40,51	5,21	31,83	10,41	17,04
4.	0,69	30,33	4,95	24,33	3,81	13,62
5.	0,50	25,75	3,01	23,59	2,97	9,32
6.	0,73	27,87	4,20	27,99	3,86	10,62
7.	1,55	37,92	4,93	23,44	6,33	13,47
8.	1,06	28,84	5,37	22,12	3,02	15,15
9.	1,28	24,20	2,31	14,70	5,89	6,98
10.	1,57	27,39	2,54	19,03	3,95	7,78
11.	1,00	19,48	2,39	19,33	3,61	7,62
12.	0,67	19,51	1,57	14,23	2,94	6,31
13.	0,59	17,53	2,00	13,17	0,91	6,10
14.	0,83	19,08	3,09	14,02	1,75	8,93
15.	1,58	21,50	2,84	3,59	5,05	10,39
MEAN F-VALUE:	0,98	25,49	3,32	19,99	3,98	9,94

APPENDIX 29.

Identification of fifteen No.10 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 10, 8, X, 9, 11 and 12.

	No.10.	No. 8.	No. X.	No. 9.	No.11.	No.12.
1.	0,70	4,23	1,25	6,63	5,62	3,32
2.	0,48	4,46	1,53	5,61	6,49	2,31
3.	1,29	10,98	2,17	5,47	5,95	4,42
4.	0,47	6,98	0,99	5,09	5,58	3,27
5.	1,28	7,44	2,20	3,65	6,34	4,11
6.	1,87	4,93	2,44	7,44	7,12	2,56
7.	0,71	10,07	1,68	4,81	6,33	4,60
8.	1,22	2,99	2,83	7,69	8,93	4,97
9.	0,66	5,94	1,58	6,06	7,73	4,91
10.	1,12	3,45	1,69	5,22	5,74	2,09
11.	1,46	12,01	2,32	5,80	8,31	5,91
12.	1,23	2,91	2,73	7,04	7,54	2,88
13.	0,54	5,21	1,16	4,67	5,49	3,78
14.	0,92	4,07	1,41	6,68	6,92	3,22
15.	0,80	8,96	1,19	4,83	6,08	3,39
MEAN F-VALUE:	1,13	6,26	1,81	5,78	6,67	3,71

APPENDIX 30.

Identification of fifteen No. 11 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 11, 10, 12, 9 and 8.

	No.11.	No.10.	No.12.	No. 9.	No. 8.
1.	0,64	16,15	9,90	2,88	13,40
2.	1,49	9,29	8,80	1,35	16,31
3.	1,55	8,15	2,57	4,27	5,07
4.	0,77	10,29	9,30	2,13	17,62
5.	0,98	17,79	12,02	3,32	12,18
6.	0,87	12,23	7,01	3,42	9,93
7.	1,01	11,36	4,69	3,19	6,87
8.	0,96	13,30	11,11	0,96	19,72
9.	0,46	9,39	5,01	2,48	9,06
10.	1,55	17,18	11,23	4,35	11,89
11.	0,92	19,14	11,81	2,79	14,38
12.	0,15	10,74	6,84	1,52	13,03
13.	1,30	15,01	11,38	1,56	20,93
14.	1,00	20,05	14,16	2,58	16,32
15.	1,05	19,20	14,06	1,50	18,72
MEAN F-VALUE:	0,95	12,67	9,32	2,55	13,66

APPENDIX 31.

Identification of fifteen No. 12 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 12, 11, 10 and 9.

	No.12.	No.11.	No.10.	No. 9.
1.	0,72	4,35	2,07	3,84
2.	0,92	4,32	5,88	6,44
3.	1,33	3,21	4,78	4,84
4.	0,34	3,21	4,22	4,31
5.	0,80	4,40	2,86	4,97
6.	1,04	4,67	4,85	5,09
7.	1,49	4,30	3,37	4,38
8.	1,96	6,65	4,23	6,20
9.	1,15	3,25	5,18	3,70
10.	1,11	5,13	7,04	6,94
11.	0,98	3,55	6,06	5,11
12.	0,79	5,50	4,50	5,04
13.	1,49	4,90	2,26	5,11
14.	1,61	6,80	5,93	5,54
15.	0,18	3,90	2,85	4,97
MEAN F-VALUE:	1,06	4,55	4,41	5,10

APPENDIX 32.

Identification of fifteen X chromosomes by computor program CROTES.

F-value of patterns tested against the mean values of chromosomes X, 7, 8, 9 and 10.

	No. X.	No. 7.	No. 8.	No. 6.	No. 9.	No.10.
1.	0,35	0,93	5,79	2,49	2,96	1,83
2.	0,59	0,74	4,35	1,78	3,21	2,53
3.	0,86	1,64	6,85	3,15	4,78	0,66
4.	1,02	1,98	3,96	2,15	5,91	0,91
5.	0,84	1,75	7,35	3,56	4,37	0,60
6.	1,18	1,45	8,55	3,99	4,15	1,94
7.	0,90	1,03	2,44	1,09	4,87	1,84
8.	0,60	1,54	4,52	2,08	5,06	0,56
9.	0,77	0,57	5,15	2,02	3,13	4,09
10.	1,36	1,40	8,14	4,02	3,01	3,20
11.	1,16	1,40	5,29	2,93	2,77	5,84
12.	0,64	2,13	8,43	3,37	1,54	9,86
13.	1,17	0,86	5,36	2,04	3,91	3,54
14.	0,38	1,33	4,01	1,78	4,48	1,09
15.	1,43	1,31	12,05	5,18	3,25	2,41
MEAN						
F- VALUE:	0,88	1,33	6,15	2,77	3,82	2,72

APPENDIX 33.

Identification of fifteen No. 13 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 13, 14 and 15.

	No. 13.	No. 14.	No. 15.
1.	0,24	7,30	4,70
2.	1,23	8,09	4,07
3.	0,85	6,69	5,21
4.	0,60	7,03	4,08
5.	0,94	8,41	6,60
6.	1,55	10,65	5,20
7.	3,05	19,57	9,81
8.	0,64	7,79	4,50
9.	0,73	7,84	5,96
10.	1,89	18,24	10,49
11.	0,49	6,49	4,43
12.	1,03	5,35	3,27
13.	0,71	5,39	4,80
14.	0,97	12,15	6,12
15.	0,45	9,90	5,88
MEAN F-VALUE:	0,94	9,38	5,70

APPENDIX 34.

Identification of fifteen No. 14 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 14, 15 and 13.

	No. 14.	No. 15.	No. 13.
1.	0,77	2,57	4,95
2.	1,58	1,47	8,50
3.	0,61	1,03	9,39
4.	1,77	2,37	6,48
5.	1,71	2,90	18,90
6.	0,94	1,09	14,23
7.	0,97	2,11	7,69
8.	0,94	1,02	8,29
9.	0,79	1,82	5,97
10.	0,97	0,55	9,11
11.	1,00	1,51	6,12
12.	0,54	0,69	6,99
13.	0,62	1,33	6,00
14.	0,82	0,56	8,35
15.	1,01	1,19	8,03
MEAN F-VALUE:	1,10	1,48	8,58

APPENDIX 35.

Identification of fifteen No. 15 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 15, 14 and 13.

	No. 15.	No. 14.	No. 13.
1.	1,11	3,11	16,26
2.	0,32	1,73	10,35
3.	2,45	8,80	22,66
4.	0,32	1,75	8,96
5.	0,66	1,19	8,23
6.	0,53	2,13	6,47
7.	1,42	2,31	5,27
8.	1,22	3,23	8,42
9.	1,18	3,74	6,24
10.	0,65	3,07	14,29
11.	1,12	3,33	8,03
12.	1,49	4,85	19,89
13.	1,18	4,97	19,72
14.	0,27	1,00	8,51
15.	0,77	1,40	7,55
MEAN F-VALUE:	0,97	3,10	11,40

APPENDIX 36.

Identification of fifteen No. 16 chromosomes by computor program CROTES.

F-value of patterns tested against the mean values of chromosomes 16, 17 and 18

	No. 16.	No. 17.	No. 18.
1.	1,00	2,40	9,42
2.	0,85	1,39	8,84
3.	1,11	1,89	7,76
4.	0,70	1,25	6,07
5.	1,76	2,68	13,95
6.	0,90	1,32	5,74
7.	0,75	0,73	3,19
8.	0,91	0,67	3,72
9.	0,54	0,65	6,35
10.	0,92	1,34	8,27
11.	1,97	1,87	5,35
12.	0,57	0,82	5,24
13.	1,74	1,75	7,81
14.	0,55	0,53	4,22
15.	0,84	1,47	8,04
MEAN F-VALUE:	1,01	1,38	6,93

APPENDIX 37.

Identification of fifteen No. 17 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes 17, 18 and 16.

	No. 17.	No. 18.	No. 16.
1.	1,43	3,94	1,86
2.	0,90	3,68	1,02
3.	1,18	6,17	1,33
4.	0,66	2,19	1,39
5.	1,96	11,57	3,41
6.	0,66	4,21	1,04
7.	1,09	5,00	1,55
8.	1,05	6,86	1,27
9.	1,72	10,59	2,63
10.	0,73	2,28	1,59
11.	0,50	3,95	0,83
12.	0,60	5,68	1,26
13.	0,68	3,18	1,03
14.	0,38	2,98	0,98
15.	0,40	2,94	1,14
MEAN F- VALUE:	0,96	5,01	1,48

APPENDIX 38.

Identification of fifteen No. 18 chromosomes by computer program CROTES.

F-value of patterns tested against the mean values of chromosomes Nos. 17, 18 and 16.

	No. 18.	No. 17.	No. 16.
1.	1,04	3,27	5,76
2.	0,97	3,01	4,75
3.	0,75	1,93	3,92
4.	1,69	2,01	4,16
5.	1,52	3,88	5,95
6.	0,39	1,86	3.91
7.	1,33	1,47	2,60
8.	0,68	1,18	2,93
9.	0,39	2,20	4,31
10.	0,59	2,91	5,43
11.	0,50	2,26	4,31
12.	3,01	3,80	5,95
13.	1,18	3,17	5,58
14.	0,37	1,72	4,18
15.	0,29	1,80	3,54
MEAN F-VALUE:	0,99	2,43	4,48

APPENDIX 39.

COMPUTER PROGRAM A V T E S T.

```
1       DIMENSION CHR(20,20),VAR(20,20),FT(20,20),FOUT(20,20),IG(20,20),FK
       2(20,20)
2       READ(5,5)((CHR(I,J),J=1,20),I=1,19),((VAR(I,J),J=1,20),I=1,19)
3     5 FORMAT(20F3.0)
4       DO 12 K=1,19
5       DO 10 I=1,19
6       SOM=0
7       VERS=0
8       DO 8 J=1,20
9       VERS=(CHR(K,J)-CHR(I,J))**2/VAR(K,J)
10      SOM=SOM+VERS
11    8 CONTINUE
12      FK(K,I)=SOM
13    10 CONTINUE
14    12 CONTINUE
15      DO 15 K=1,19
16      DO 13 I=1,19
17      FOUT(K,I)=FK(K,I)
18    13 CONTINUE
19    15 CONTINUE
20      DO 40 K=1,19
21      DO 30 L=1,19
22      GRTS=1000.
23      DO 25 I=1,19
```

Appendix 39 continued.

```
24          IF(FOUT(K,I).LE.GRTS)GO TO 20
25          GO TO 25
26       20 GRTS=FOUT(K,I)
27          KN=I
28       25 CONTINUE
29          I=KN
30          FOUT(K,I)=1000.
31          IG(K,L)=I
32          FT(K,L)=FK(K,I)
33       30 CONTINUE
34       40 CONTINUE
35          WRITE(6,41)
36       41 FORMAT('1',4X,' FOUT 1 / ',5X,' FOUT 2 / ',5X,' FOUT 3 / ',5X,' FO
          2UT 4 / ',5X,' FOUT 5 / ',5X,' FOUT 6 / ',5X,' FOUT 7 / '//)
37          DO 50 J=1,19
38          WRITE(6,45)IG(1,J),FT(1,J),IG(2,J),FT(2,J),IG(3,J),FT(3,J),IG(4,J)
          2,FT(4,J),IG(5,J),FT(5,J),IG(6,J)FT(6,J),IG(7,J),FT(7,J)
39       45 FORMAT(7(5X,12,F8.2)//)
40       50 CONTINUE
41          WRITE(6,51)
42       51 FORMAT(5X,' FOUT 8 / ',5X,' FOUT 9 / ',5X,' FOUT 10/ ',5X,' FOUT 1
          21/ ',5X,' FOUT 12/ ',5X,' FOUT 13/ ',5X,' FOUT 14/ ')
43          DO 60 J=1,19
44          WRITE(6,65)IG(8,J),FT(8,J),IG(9,J),FT(9,J),IG(10,J),FT(10,J)?IG(11
          2,J),FT(11,J),IG(12,J),FT(12,J),IG(13,JFT(13,J),IG(14,J),FT(14,J)
45       65 FORMAT(7(5X,12,F8.2)//)
46       60 CONTINUE
47       WRITE(6,61)
```

Appendix 39 continued.

```
48          61 FORMAT(5X,' FOUT 15/ ',5X,' FOUT 16/ ',5X,' FOUT 17/ ',5X,' FOUT
              28/ ',5X,' FOUT 19/ ')
49             DO 70 J=1,19
50             WRITE(6,75)IG(15,J),FT(15,J),IG(16,J),FT(16,J),IG(17,J),FT(17,J),I
              2G(18,J),FT(18,J),IG(19,J),FT(19,J)
51          75 FORMAT(7(5X,12,F8.2)//)
52          70 CONTINUE
53             CALL EXIT
54             END
```

APPENDIX 40.

COMPUTER PROGRAM C R O T E S.

```
1.        DIMENSION CHR(20,20),VAR(20,20),FT(500,25),FOUT(500,20),CHRT(500,2
         20),IG(500,20),FK(500,20)
2.        READ(5,4)N
3.      4 FORMAT(13)
4         READ(5,5)((CHR(I,J),J=1,20),I=1,19),((VAR(I,J),J=1,20),I=1,19),((C
         2HRT(I,J),J=1,20),I=1,N)
5       5 FORMAT(20F3.0)
6         DO 12 I=1,N
7         DO 10 K=1,19
8         SOM=0
9         VERS=0
10        DO 8 J=1,20
11        VERS=(CHR(K,J)-CHRT(I,J))**2/VAR(K,J)
12        SOM=SOM+VERS
13      8 CONTINUE
14        FK(I,K)=SOM/19
15     10 CONTINUE
16     12 CONTINUE
17        DO 15 I=1,N
18        DO 13 K=1,19
19        FOUT(I,K)=FK(I,K)
20     13 CONTINUE
21     15 CONTINUE
22        DO 40 I=1,N
23        DO 30 L=1,19
```

Appendix 40 continued.

```
24          GRTS=100.
25          DO 25 K=1,19
26          IF(FOUT(I,K).LE.GRTS)GO TO 20
27          GO TO 25
28       20 GRTS=FOUT(I,K)
29          KN=K
30       25 CONTINUE
31          K=KN
32          FOUT(I,K)=100
33          IG(I,L)=K
34          FT(I,L)=FK(I,K)
35       30 CONTINUE
36       40 CONTINUE
37          WRITE(6,103)
38      103 FORMAT('1',/////)
39          DO 110 I=1,N
40          J=1
41          WRITE(6,104)I,IG(I,J),FT(I,J
42      104 FORMAT(2X ,13,'.',5X'THE CHROMOSOME UNDER TEST IS PROBABLY NO.',
           213,'(F   =',F8.5,') WITH THE FOLLOWING POSSIBILITIES IN ORDER OF
           3',/' RANK:(F VALUE IN PARENTHESIS)'/)
43          DO 108 J=2,21,5
44          WRITE(6,106)IG(I,J),FT(I,J),IG(I,J+1),FT(I,J+1),IG(I,J+2),FT(I,J+2
           2),IG(I,J+3),FT(I,J+3),IG(I,J+4),FT(I,J+4)
45      106 FORMAT(5(2X,13,5X,'(',F8.5,')'))
46      108 CONTINUE
47      110 CONTINUE
48          CALLEXIT
49          END
```

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